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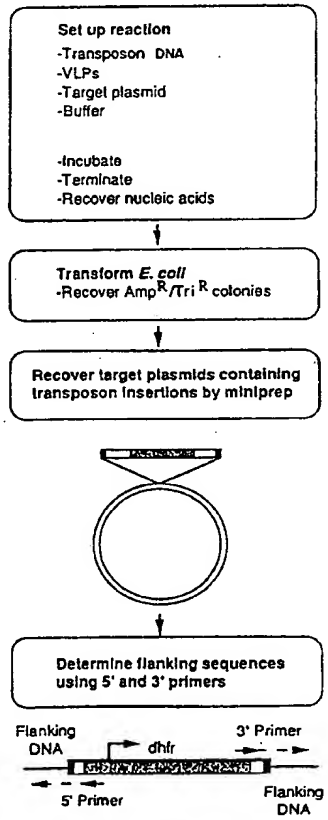
(57) Abstract

We have developed efficient methods of creating artificial transposons and inserting these transposons into DNA targets *in vitro*, primarily for the purpose of mapping and sequencing DNA. A target DNA has been engineered to convert virtually any DNA sequence, or combination of sequences, into an artificial transposon; hence, custom transposons containing any desired feature can be easily designed and constructed. Such transposons are then efficiently inserted into DNA targets, *in vitro*, using the integrase activity present in yeast Ty1 virus-like particles. Primers complementary to the transposon termini can be used to sequence DNA flanking any transposon insertion.



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IN VITRO TRANSPOSITION OF ARTIFICIAL TRANSPOSONS

BACKGROUND OF THE INVENTION

DNA sequencing has helped revolutionize the way that genes and genomes are studied, and has led to a greater understanding of most aspects of biology. Nevertheless, with efforts underway to map and sequence the genomes of a variety of organisms, the need to improve the efficiency of DNA sequencing has never been greater (1). One of the major problems associated with sequencing large segments of DNA is obtaining sequence information beyond the limits of a single primer extension event. Several techniques are currently used to acquire sequences within the interior of a DNA insert; these include: i) the synthesis of custom primers to "walk" along a segment of DNA (2, 3), ii) shotgun subcloning, which requires a high degree of redundancy for complete sequence recovery (4), or iii) the construction of overlapping exonuclease deletion clones (3, 5). Each of these methods is time-consuming, idiosyncratic and therefore difficult to automate, and/or costly.

Alternatively, transposable elements have been adapted for DNA mapping and sequencing. Examples include: $\gamma\delta$ (6), Tn5 (7), Tn10 (8), as well as derivatives of these and other transposons. Although these approaches generally offer great promise, the insertion step is performed *in vivo* in *E. coli*; hence,

transposition may occur into either the plasmid target or the *E. coli* genome, complicating the recovery of target insertions. An additional difficulty arises from host effects on insertion randomness, i.e., "hotspots" and "coldspots" of integration are often observed *in vivo* (9).

The complete DNA integration reaction employed by certain retroviruses and retrotransposons as part of their normal life cycles can be carried out completely *in vitro* (10-14) offering a possible alternative to *in vivo* transposon insertion techniques for DNA sequencing.

There is a need in the art for a simple, reliable technique for generating sets of DNA templates for sequencing any target. In particular there is a need for sets of DNA templates which are amenable to automated sequencing with a single set of primers.

SUMMARY OF THE INVENTION

It is an object of the invention to provide methods for providing templates for DNA sequencing.

It is another object of the invention to provide methods for sequencing such DNA templates.

It is yet another object of the invention to provide a kit for DNA sequencing.

It is yet another object of the invention to provide an artificial transposon.

It is still another object of the invention to provide plasmids for preparing artificial transposons.

It is yet another object of the invention to provide methods for the generation *in vitro* of insertions into a target DNA molecule.

These and other objects of the invention are provided by one or more of the embodiments of the invention described below. In one embodiment a method is provided for preparing templates for DNA sequencing. The method comprises the steps of:

incubating *in vitro*: (1) a population of a target DNA, said target DNA comprising a region of DNA to be sequenced, (2) a retroviral or

retrotransposon integrase, and (3) an artificial transposon having two termini which are substrates for said integrase, wherein the molar ratio of artificial transposon to target DNA is at least 1:1, to form a population of target DNAs with quasi-randomly integrated insertions of the artificial transposon;

transforming host cells with the population of target DNAs with quasi-randomly integrated insertions of the artificial transposon;

selecting those host cells which have been transformed with a target DNA with an insertion of the artificial transposon;

isolating target DNA with an insertion of the artificial transposon from those host cells which have been transformed with a target DNA with an insertion of the artificial transposon, said target DNA with an insertion of the artificial transposon being suitable for use as a DNA sequencing template.

In another embodiment a method is provided for sequencing DNA. The method comprises the steps of:

incubating *in vitro* (1) a population of a target DNA, said target DNA comprising a region of DNA to be sequenced, (2) a retrovirus or retrotransposon integrase, and (3) an artificial transposon having two termini which are substrates for said integrase, wherein the molar ratio of artificial transposon to target DNA is at least 1:1, to form a population of target DNAs with quasi-randomly integrated insertions of the artificial transposon;

transforming host cells with the population of target DNAs with quasi-randomly integrated insertions of the artificial transposon;

selecting those host cells which have been transformed with a target DNA with an insertion of the artificial transposon;

isolating target DNA with an insertion of the artificial transposon from those host cells which have been transformed with a target DNA with an insertion of the artificial transposon, said target DNA with an insertion of the artificial transposon being suitable for use as a DNA sequencing template;

hybridizing to said isolated target DNA with an insertion of the artificial transposon a primer which is complementary to a terminus of the artificial transposon;

extending said primer to determine a nucleotide sequence of DNA flanking said artificial transposon in said isolated target DNA with an insertion of the artificial transposon.

In still another embodiment of the invention a method for sequencing DNA is provided. The method comprises the steps of:

providing a population of target DNAs with quasi-randomly integrated insertions of an artificial transposon, said artificial transposon having termini which are substrates for a retrovirus or a retrotransposon, said population of target DNAs having been formed by *in vitro* insertion of said artificial transposon into the target DNAs using a retroviral or retrotransposon integrase and a molar ratio of artificial transposon to target DNA of at least 1:1;

hybridizing to individual target DNAs of said population a primer which is complementary to a terminus of the artificial transposon;

extending said primer to determine a nucleotide sequence of target DNA flanking said artificial transposon.

In still another embodiment of the invention a kit for DNA sequencing is provided. The kit comprises:

an artificial transposon having termini which are substrates for a retroviral or retrotransposon integrase;

a retroviral or retrotransposon integrase;

a buffer for *in vitro* transposition of said artificial transposon, said buffer having a pH of 6 to 8 and 1 to 50 mM of a divalent cation; and

a primer which is complementary to a terminus of said artificial transposon.

In an additional embodiment of the invention an artificial transposon is provided. The transposon consists of a linear DNA molecule comprising:

a marker gene;

a sequence of yeast retrotransposon Ty1, said sequence selected from the group consisting of a U5 sequence and a U3 sequence, said sequence flanking said marker gene on its upstream end, said sequence consisting of 4 to 11 bp of terminal sequences of said Ty1; and

a sequence of yeast retrotransposon Ty1, said sequence selected from the group consisting of a U5 sequence and a U3 sequence, said sequence flanking said marker gene on its downstream end, said sequence consisting of 4 to 11 bp of terminal sequences of said Ty1.

In yet an additional embodiment of the invention a DNA molecule useful for generating artificial transposons is provided. The DNA molecule comprises:

an origin of replication;

a first selectable marker DNA;

two blunt-ended transposon termini of at least 4 bp each, said termini being substrates for yeast retrotransposon Ty1 integrase, said transposon termini flanking a first restriction enzyme site useful for insertion of a second selectable marker gene to form an artificial transposon;

a second restriction enzyme site flanking said two transposon termini, wherein digestion with said second restriction enzyme liberates a blunt-ended fragment having said transposon termini at either end of the fragment, the fragment thereby liberated being an artificial transposon.

In still another embodiment of the invention a method for *in vitro* generation of insertions into a target DNA is provided. The method comprises the steps of:

incubating *in vitro* (1) a population of a target DNA, (2) a retroviral or retrotransposon integrase, and (3) an artificial transposon having termini which are substrates for said integrase, wherein the molar ratio of artificial transposon to target DNA is at least 1:1, to form a population of target DNAs with quasi-randomly integrated insertions of the artificial transposon;

transforming a host cell with the population of target DNAs with quasi-randomly integrated insertions of the artificial transposon;

selecting those host cells which have been transformed with a target DNA with an insertion of the artificial transposon.

The *in vitro* systems of the present invention offer several advantages over *in vivo* transposition systems: i) special bacterial strains are not required, ii) potential host effects are avoided, and iii) an *in vitro* reaction is amenable to biochemical alteration and parameter optimization. Thus a simple and reliable method is provided for generating large amounts of sequence information, such as is required for sequencing of entire genomes of particular organisms.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Overview of artificial transposon insertion into plasmid targets.

The basic steps involved in generating artificial transposon insertions in target plasmids are indicated. Note the following: DNA sequences to be determined (dashed line) trimethoprim resistance (tri^r) gene (shaded box); target plasmid (double circle); PART (primer island artificial transposon) (box); Ty1 U3 termini (filled rectangles).

Figure 2. pAT-1 and pAT-2.

Fig 2A. The backbone common to pAT-1 and pAT-2 is shown to contain the yeast *URA3* gene, a bacterial origin of replication (ori) and a multicloning site (mcs). pAT-2, containing the PART insert, is depicted. Fig 2B. The PART which is created upon digestion with *Xmn* I, is shown. It contains the dhfr (dihydrofolate reductase) gene (stippled), the pBLUESCRIPT mcs (white boxes), and Ty1 U3 cassettes (filled rectangles), as well as two unique primer sites for sequencing the DNA flanking an insertion site. Fig 2C. The sequence at Ty1 U3/ *Xmn* I cassettes. The arrows indicate the *Xmn* I cleavage site. The shaded areas indicate Ty1 U3 sequences (one on either side of the arrows), while the entire sequence encodes a recognition site for *Xmn* I.

Figure 3. PART insertions in clone p76-2.

The 8 kb insert of clone p76-2, containing a segment of yeast chromosome III, is shown along with the sites of 78 independent PART insertions (*arrows*). The orientation of transposon insertion is indicated: (↓) Forward (the dhfr gene

in the artificial transposon is transcribed left to right, or (↑) Reverse. This region of chr. III contained on the insert includes the *PGK* 1 gene (black box), a glycine tRNA gene (black circle with arrowhead indicating direction of transcription), a Ty1 solo delta (stippled box) and the *YCR16w* locus (striped box). The PART insertion locations were determined by sequencing one or both insertion junctions.

Figure 4. Conceptual contig map.

The locations of the 78 PART insertions were used to construct a conceptual contig map based on the following assumptions: i) two primer extensions would be initiated from each PART (one in each direction) and ii) each extension would lead to the recovery of 250 bp of useful DNA sequence information.

Figure 5. Interval Sizes of PART insertions into p76-2.

The size of intervals between individual insertions of PART into p76-2 (*i.e.*, the distance between adjacent insertions in bp) were grouped and the number of intervals falling within each group is graphically represented.

Figure 6. Distribution of PART insertions in plasmid pWAFp.

Plasmid pWAFp contains a 5 kb insert of human DNA encoding the WAF-1 promoter. We generated PART insertions into this target using an artificial transposon prepared by PCR and digestion with *Bbs* I to generate U3 and U5 sequences at the upstream and downstream ends of the transposon, respectively. Of 45 insertions analyzed, 12 mapped to the pBLUESCRIPT vector fragment (shown in black), 13 mapped to the 1.5 kb *Not* I/*Pst* I fragment of the WAF-1 insert, 12 mapped to the 2.5 kb *Pst* I fragment of WAF-1 (WAF-1 sequences are solid white). Hence, insertions were recovered from all regions of this target plasmid, and the insertion frequencies ranged from 4.1 insertions/kb to 10 insertions/kb target DNA. This set of insertions was then used to directly recover greater than 90% of the WAF-1 DNA sequence.

Figure 7. Distribution of insertions into yeast chromosome III.

An artificial transposon having one U3 and one U5 terminus, each 4 pb in length, was generated by PCR, digested with *Bbs* I, and filled-in with Klenow fragment of DNA polymerase I. Distribution of insertions are shown on a map of the chromosome III segment of DNA contained on the target plasmid.

Figure 8A-8B. The nucleotide sequence of pAT-1.

Figure 9A-9B. The nucleotide sequence of pAT-2.

Figure 10. The nucleotide sequence of the PART from pAT-2.

Figure 11. Sequence contig map for 8 kb region of cosmid F13544.

169 independent AT-2 insertions were generated in the cosmid F13544 by *in vitro* integration. A collection of 43 insertions which were found to map to an 8 kb region by restriction mapping were assembled and sequenced using primers SD118 and 119 in conjunction with ABI Prism technology. A contig map of the sequencing project is indicated. Each arrow represents a single primer extension event. Beneath is a map of sequence completion. Black areas indicate sequence on both strands, whereas hatched areas are on one strand only.

Figure 12. Artificial transposons.

Eight different artificial transposons, including the AT-1 sequence and structure, are shown. Each was derived from either pAT-1 or pAT-2, and is prepared from its plasmid with the same Xmn I strategy used for these plasmids.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

It is a discovery of the present invention that a transposon insertion technique that is carried out entirely *in vitro* may be applied to a variety of problems, including DNA sequencing. This technique employs artificial transposons which are created using a plasmid construct, and retroviral or retrotransposon integrase, which may be provided in the form of viral or virus-like particles (VLPs), which mediates the insertion of these transposons into target DNA molecules.

We have developed new methods for creating artificial transposons and efficiently inserting these transposons into DNA targets, *in vitro*. There are three key aspects of the process: i) the *in vitro* integration reaction is highly efficient, giving rise to thousands of integrations per reaction; with most plasmid targets, this efficiency approaches one insertion per phosphodiester bond, ii) the insertion process is sufficiently random that transposon integrations occur throughout target plasmid sequences, and iii) virtually any DNA sequence or combination of sequences can, in principle, serve as an artificial transposon. These three features combine to make this an extremely versatile method of generating recombinant DNA molecules.

Artificial transposons are ideal for DNA sequencing: i) a large number of transposon insertions can be easily assembled from a single integration reaction, allowing the recovery of insertions suitably spaced to facilitate sequencing of a DNA segment, ii) the transposon can be engineered to contain desired features useful for DNA mapping or sequencing, and iii) since each transposon carries two unique primer sites, the nucleotide sequence flanking each insertion site can be rapidly and efficiently determined. A set of plasmids bearing artificial transposon insertions are especially useful for sequencing because all the plasmids can be sequenced in parallel using a defined pair of primers. This is in contrast to the inefficient "series" approach of primer walking, in which each sequence is used to specify the next primer. Hence, artificial transposons are flexible and extremely efficient for generating DNA sequencing templates useful for both small and large-scale DNA sequencing projects.

There are three macromolecular components to the *in vitro* integration reaction: i) an artificial transposon, ii) retroviral or retrotransposon integrase and iii) a DNA target. These three components are mixed together in a reaction containing the appropriate buffer and cofactors. In the case of yeast retrotransposon Ty1, the reaction is briefly incubated at 30° and 37° Celsius, and terminated by adding EDTA and heating to 65° Celsius. Finally, the nucleic acids are phenol/chloroform extracted and ethanol precipitated. The recovered DNA is

used to transform a host cell to drug resistance (or other suitable selectable marker), allowing the identification of target molecules which have received a transposon integration (Fig. 1). A set of transposon-bearing target DNA molecules may then be used directly to obtain the DNA sequences flanking the insertion sites, using two primers corresponding to the transposon termini; a collection of such insertions can be used for the efficient recovery of DNA sequence information from the region of interest.

We have focused our initial efforts on developing a specific application of this technology, i.e., *in vitro* insertion of "primer island" artificial transposons (PARTs) into plasmid targets for the purpose of DNA mapping and sequencing. In addition to the features mentioned above (efficiency of integration, randomness of insertion, and flexibility of transposon), this system has other advantages compared with existing methods, including: i) the *in vitro* protocol is simple and highly reliable, even in the hands of a novice, ii) the PART does not contain large terminal repeats which, in Tn5 and Tn10-based systems, hinder access to sequences flanking the insertion junctions, and iii) the reaction is carried out completely *in vitro* and therefore is amenable to biochemical alteration and parameter optimization; this may be especially useful with unusual DNA templates such as those containing tandem sequence repeats, high GC content, or unusual template topology which might represent difficult targets.

Importantly, transposon integration within targets was sufficiently random that insertions were recovered from all regions of target DNAs. Hence, Ty1 integrase-mediated integration *in vitro* is, at a minimum, a nearly-random process. It may, in fact, be totally random. This will only become clear upon testing large numbers of targets containing different DNA sequence features. Nevertheless, our current results strongly support a model of quasi-random insertion with no apparent major biases. In contrast, this feature is not generally observed of other transposon systems adapted for DNA sequencing; instead, hotspots and coldspots of insertion frequently lead to a non-random distribution of insertions rendering these systems incapable of accessing large segments of DNA sequence, or high

levels of wasteful redundancy in other regions. These problems have been circumvented in some systems with mutant transposases which display altered target specificity (9). However, this approach provides only a limited relaxation of transposase-specified target specificity. It is known that host cell factors contribute to target specificity *in vivo* for both Tn10 (9, 9a) and Ty1 (28); such target specificity is eliminated by the use of *in vitro* systems as taught herein. Fortunately, the process of artificial transposon integration *in vitro* by retroviral and retrotransposon integrases, such as Ty1 integrase, displays random-like behavior (Fig. 2), making it ideal for the purpose of DNA sequencing. Quasi-random, according to the present invention, means that insertions can be obtained in virtually any sequence at a spacing of at least one integration per kb. In practice, integrations have been obtained at maximum spacings of as low as one integration per 500 bp, or even one integration per 400 bp. In contrast, large cold-spots have been found in targets of Ty1 transposition *in vivo*.

Because our method of constructing artificial transposons is very versatile, transposons containing a variety of sequences can be constructed for a number of specific applications. For example, other markers can be inserted into the multicloning site (mcs) site of pAT-1, including but not limited to yeast and mammalian drug-selectable or auxotrophic genes, generating marker cassettes that can act as transposons. Such artificial transposons can be used for "marker addition", i.e., the insertion of a useful auxotrophic marker into an acceptable region of a plasmid of interest. For use in bacteria or yeast, for example, pAT-1 derivatives containing a variety of selectable markers in the mcs can be constructed, and the marker of choice (auxotrophic, drug resistance, suppressor, etc.) can be added to a target plasmid with a simple *in vitro* integration reaction. Indeed, the products of a single integration reaction can be viewed as an "integration library" containing a collection of insertions, each clone containing a single insertion at a particular phosphodiester bond. Should it be necessary, an insertion at any specific phosphodiester bond can be identified with conventional library screening methods, using a junction oligonucleotide as a probe. Hence,

using a custom artificial transposon, and applying the appropriate screening method, recombinant molecules of a desired structure can be recovered.

In addition to the artificial transposon, the other two components of the system, i.e., the integrase and the target, are also versatile. For example, other integrases or transposases can effect an equivalent or nearly-equivalent *in vitro* integration reaction. In addition, mutant integrases are also useful. The specific properties of such integrases might together provide a wider range of integration preferences or frequencies. Also, rather than providing the integrase in the form of viral particles or VLPs, purified integrases can be used. These may display altered levels of activity or stability, relative to VLP-associated integrases.

The *in vitro* integration reaction can employ a variety of DNA targets. Plasmids, including cosmids, artificial chromosomes, as well as bacteriophage or viral vectors are useful. Bacteriophage lambda DNA has been used as a target in similar reactions using Moloney murine leukemia virus (10) and Ty1 integrases (11,12) provided in the form of viral particles.

The PART-based system for generating DNA sequencing templates can be readily applied to the development of high throughput, massively parallel DNA sequencing strategies. The high degree of randomness of insertion and the large fraction of clones generating useful sequence data mean that a shotgun approach to sequencing of large recombinant plasmids, including cosmids as well as P1 and bacterial artificial chromosomes, is feasible and highly suited to automation. Random doubly drug resistant colonies can be selected, their DNA extracted, and fed directly into an automated sequencing apparatus. All of these steps are amenable to automation. Because a single set of optimized primers can be used to sequence an entire set of plasmid derivatives, all of the steps can be done in parallel without operator intervention with regard to primer design and selection, etc. Hence, although artificial transposon-facilitated DNA sequencing is predicted to be very useful for small-scale sequencing projects, it may be even more useful for massive projects such as the effort underway to map and sequence the human genome.

The artificial transposon which is employed according to the present invention contains a 3'-hydroxyl and is blunt-ended. Such molecules can be prepared using restriction enzymes which make staggered cuts followed by a "filling-in" reaction with a DNA polymerase, such as Klenow fragment of DNA polymerase I. Alternatively, the artificial transposon can be prepared by a PCR. Typically the ends of PCR products require "trimming" to generate blunt ends. Thus a restriction enzyme, such as *Xmn* I, which makes blunt-ended termini can be used to trim a PCR product. Most simply, an artificial transposon contained in a plasmid can be isolated from the plasmid with a restriction enzyme, such as *Xmn* I, which makes blunt-ended termini. This provides a homogenous preparation of blunt-ended fragments in one step.

Integrase activity can be provided by virus-like particles, in the case of yeast retrotransposon Ty1, or by cellular nucleoprotein complexes in the case of retroviral particles. Alternatively, purified integrase may be used. It is desirable that the artificial transposon be added to the *in vitro* transposition incubation mixtures as protein-free DNA preparations. Although some native transposon DNA may be present in the integrase preparations, typically such transposons will not be genetically marked, and will be present in significantly lower molar amounts than the artificial transposon.

DNA contained within a transposon's termini may be any desirable marker or even a cryptic sequence. Antibiotic resistance genes, useful for either prokaryotes or eukaryotes are often useful. Auxotrophic markers are also useful, especially in yeast. Cis-acting regulatory elements, such as promoters, may also be desired to ascertain function of previously unknown regions flanking an insertion. Marker DNAs also includes other non-coding features, such as restriction sites, primer binding (hybridization) sites, etc.

The ratio of artificial transposon to target DNA has been found to be a significant factor in the efficiency of the reaction. Desirably the molar ratio will be at least 1:1, and more preferably the molar ratio will be at least 2.5:1, 10:1 or 50:1.

Host cells may be transformed by any means known in the art, including transfection, transduction, electroporation, etc. Selection of transformed cells is typically and conveniently carried out by a genetic selection means, although genetic and biochemical screening methods may also be employed.

In the case of Ty1 transposition, the use of the entire U3 or U5 terminal sequences has been found to be unnecessary. Thus as little as 4 bp of terminal sequence of U3 and/or U5 can be used. (The sequence of U3 and U5 are disclosed in figure 5 of reference 12.) While there is some evidence that other unrelated sequences may be suitable as a substrate for integrase enzymes to generate single transposon-end joining products (14), such sequences may not be suitable for generating the two transposon-end, complete integration product necessary for the present invention.

Primers which are employed for sequencing according to the present invention are those which are known in the art for dideoxy-type sequencing. These are typically synthetic, single-stranded oligonucleotides of about 12-60 bases in length. It is desirable, according to the present invention that the primers for sequencing each flank of the inserted transposon be unique. Therefore, if the two transposon termini are identical, which they can be, the primer complementarity must extend into or be wholly derived from the "marker region" so that each primer only hybridizes to a single end of the transposon. Primers "complementary to a terminus of an artificial transposon" are those oligonucleotides which are 12 to 60 bases in length which are derived from the terminal approximately 150 bp of the artificial transposon. Primer sequences which are optimized for DNA sequencing can easily be designed into the artificial transposon.

Viral particles, according to the present invention are nucleoprotein complexes which are isolated from cellular extracts of infected cells. In the case of yeast retrotransposon Ty1, the particles are known as virus-like particles. An integrase activity can be purified from such particles using protein purification techniques known in the art. While Ty1 is exemplified in this application, it is believed that its closely related yeast retrotransposon Ty2 will be equally useful.

In addition, retroviral and other integrases may also be used according to the present invention. Avian myeloblastosis virus (AMV) integrase can be used to mediate the concerted integration of an artificial transposon into a target DNA (30). Murine leukemia virus (MLV) and human immunodeficiency virus (HIV) retroviral integrases mediate quasi-random insertion of artificial transposons into target DNAs (31). The 3-D structure of HIV-1 integrase core domain has been shown to be similar to the bacterial transposase, MuA (32). Thus bacterial transposases could also be used in a similar manner.

It has been found that divalent cations are necessary for transposition. Suitable concentrations of magnesium or manganese ions range from about 1 to about 50 mM. Preferably the concentration is between about 5 and 45 mM. The pH range which is suitable for *in vitro* transposition is broad, from pH 6 to 8, and may desirably be from pH 7 to pH 8.

In addition to the application of PART technology to the sequencing of DNA, there are a number of other applications which are possible, owing to the high efficiency and randomness of insertion of PARTs. Some of these are outlined below.

1. **DNA sequencing and mapping**

i) Small-scale DNA sequencing.

Example: A 3.5 kb segment of DNA is cloned into a plasmid cloning vector. The investigators wish to obtain the complete nucleotide sequence of this 3.5 kb insert, on both strands using polymerase-based (Sanger) dideoxy sequencing. PART insertions are generated throughout the plasmid *in vitro*. The collection is screened by restriction mapping to determine whether individual PART insertions are located in the plasmid backbone or the insert, and a collection of target plasmids bearing insertions every 100-200 bp in the insert is recovered. Each PART is then used to sequence the DNA on both sides of the insertion, using unique primers homologous to the termini of the PART. Since standard dideoxy sequencing protocols lead to the recovery of 200-300 bp (or more) useful sequence information, the entire sequence of the 3.5 kb insert is recovered, on both strands.

ii) Large-scale sequencing.

Example: A yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC), or other vehicle used for the propagation of large segments of DNA contains a large segment of human DNA that requires DNA sequence analysis. Assuming that a 400 kb YAC is used, the YAC is resolved on a pulsed field gel cast with low-melting point agar, and excised. PART insertions are generated *in vitro* within the YAC. A specialized PART derivative, containing a selectable yeast marker is used to enable the facile recovery of PART insertions by transforming the collection into yeast by protoplast fusion, with subsequent selection for complementation of an auxotrophy. PART insertions are recovered throughout the YAC in this manner. Each PART insertion is then used to recover sequence from the flanking DNA in both directions by cycle sequencing, using a thermostable polymerase. YACs bearing PART insertions are shotgun sequenced until the entire sequence is recovered. The original linkage of the sequence is maintained throughout the procedure, making data assimilation simpler than most large-scale sequencing methods. Finally, many aspects of this process are amenable to automation.

iii) DNA Mapping.

Using PART insertions such as those described above, a PART map could be constructed in a DNA segment of interest. Since the PART contains a number of useful restriction sites (6-bp and 8-bp cutters), the location of the insertions relative to the endpoints of the insert could be determined by cutting the clone with an enzyme such as *Not* I, and running the products on the appropriate gel. The sizes of the products would yield information about the location of the PART insertion relative to the ends and other sites such as known genes or *Not* I sites. The sequence information recovered from such a PART insertion could then be correlated with a map position. This approach enables the rapid assignment of a sequence tag to a map position, which would be a useful intermediate on the way to completing the entire sequence, especially if an entire genome is being sequenced. Another advantage is that the original linkage of the various map

positions is maintained throughout the mapping procedure. Alternatively, PCR mapping strategies can be used to map the position of the insertion, using one PCR primer corresponding to a transposon end and one primer corresponding to a known position in the target plasmid. The size of the resultant PCR products allows the insert position and orientation to be determined.

2. **Gene mapping by integrative disruption.**

Example: A yeast gene has been cloned as part of a large, e.g., 15 kb DNA insert on a plasmid. The investigator wishes to know where, within this 15 kb, the gene is located. The clone was originally isolated by complementation of a mutant phenotype in yeast; hence, a functional assay for the presence of the gene exists. A set of PART insertions is made into the target plasmid and these are then transformed into yeast; non-complementing clones should contain insertions into the gene of interest. A selectable yeast gene (e.g., *URA3, TRP1* or *HIS3*) could be incorporated into the artificial transposon, both simplifying the original selection in yeast for clones maintaining a transposon insertion, and allowing the facile identification of gene disrupter clones which could be later used directly to knock out the gene of interest in the host genome.

3. **Introduction of any functional or non-functional DNA cis element, sequence, or combination of sequences into another segment of DNA.**

i) Restriction sites for mapping, making deletions, adding new DNA fragments/sequences.

Restriction enzymes are multipurpose tools. By inserting a site for a particular enzyme at a desired location, the site could be used for mapping, making deletions or adding restriction fragments to the target DNA.

Example 1: An artificial transposon containing two *Not* I restriction sites flanking a selectable marker is inserted into the target plasmid *in vitro*. Miniprep DNAs are screened by restriction mapping to locate an artificial transposon insertion in the desired region. Alternatively, an insertion library containing artificial transposon insertions throughout the target clone is screened with a junction oligonucleotide to identify an insertion at a particular phosphodiester

bond. Once a suitably-positioned transposon is identified, the plasmid is cleaved with *Not* I, thus removing the majority of the transposon, and generating ends with a *Not* I restriction site. Since many sites flank the selectable marker in pAT-1 and pAT-2, this approach could be adapted for use with any pair of enzymes that would lead to the removal of the selectable gene and allow the subsequent cloning of an insert at the site. This general approach offers an alternative to creating a restriction endonuclease site by the method of site directed mutagenesis.

Example 2: A yeast artificial chromosome (YAC) containing 800 kb of human DNA is used as a target to generate artificial transposon insertions. Upon recovery of insertions, one is mapped to a position near a site thought to contain no functional genes. Since the artificial transposon contains a single *Not* I site and the chromosome lacks *Not* I sites, the unique site could be used to insert a novel gene into this location.

ii) Promoters, enhancers, terminators, introns, exons.

Example: An artificial transposon is created which contains the third exon of gene W which is known to encode a stretch of 99 prolines followed by 33 histidines and then 11 tyrosines. Normal mammalian 5' splice donor, 3' splice acceptor, and branch acceptor sites are incorporated into the transposon at the appropriate positions for correct splicing, along with a selectable marker. The transposon is integrated into gene X on a plasmid, and the plasmid subsequently transfected into mammalian cells in culture. The exon is found to be appropriately incorporated into the transcribed mRNA of gene X, with precise excision of all non-exon sequences. The protein chemistry of the region encoded by this exon is now studied in the new protein context.

iii) Drug-selectable or auxotrophic markers useful in experimental and non-experimental organisms including: bacteria, plants, yeast, insects, *Drosophila*, worms, rodents, humans, mammals in general.

"Marker swap" or "Marker addition" transposons.

Goal: introduce or exchange genetic markers in a vector of interest, using the integration reaction rather than restriction enzymes. Transposons similar to the

PART but containing different drug resistance (chloramphenicol, kanamycin) or yeast selectable markers (*URA3, TRP1, HIS3, LEU2*) between the transposon termini could be integrated into a target plasmid of choice. The resultant plasmids could be selected for the acquisition of the new marker and then if desired, be screened for loss of a pre-existing marker.

Example: You have a plasmid that contains a marker for ampicillin resistance as well as a gene of interest. For an upcoming experiment, you desire that the plasmid contain a chloramphenicol resistance marker, and require that the plasmid be lacking the ampicillin gene. Thus, the end goal is to have a single plasmid carrying your gene of interest, a chloramphenicol resistance marker, and no ampicillin resistance marker. To accomplish this, you perform an *in vitro* integration with an artificial transposon containing a chloramphenicol gene, and select plasmids that are chloramphenicol resistant. Next, you replica plate to ampicillin-containing plates, and identify chloramphenicol resistant/ampicillin sensitive clones. The new marker is found to have integrated within the Amp marker.

iv) Genes. Any gene of interest could be cloned into a pAT derivative and directly inserted as a transposon into a DNA target.

Example: A gene therapist wants to build a variety of new adenovirus constructs to test as delivery vehicles for the cystic fibrosis transmembrane regulator (CFTR) gene, which is the human gene responsible for cystic fibrosis. Since both the adenovirus genome and the CFTR cDNA are both quite large, strategies based on restriction enzymes are not easily identified. Instead, the gene therapist clones the CFTR cDNA driven by the CFTR promoter into a pAT derivative carrying a selectable marker, and inserts the resultant artificial transposon carrying the CFTR gene into the adenovirus vector. Thus, various constructs are rapidly built and tested.

v) Any functional or non-functional DNA

DNA segments comprised of any nucleotide sequence or combination of sequences, could be envisioned to be incorporated into an artificial transposon, thus becoming amenable to recombination with a target via an integration reaction.

vi) Codon insertion mutagenesis.

Restriction sites for a rare cutting restriction enzyme (e.g. SrfI, cutting GCCCGGGC) can be positioned just inside the termini of the artificial transposon, but flanking the selectable marker (e.g. dhfr). The restriction sites can be positioned such that, after deletion of the marker-containing (dhfr in the example) SrfI fragment, there would be a net insertion of an integral number of codons into the target plasmid, resulting from the new bases introduced (these would consist of the target site duplication, artificial transposon terminal base pairs, and the restriction site, plus one or two additional base pairs as necessary to ensure the proper reading frame). Following insertion of such an artificial transposon into a target plasmid or cosmid of interest, the population of insertion mutant plasmids or cosmids could be digested en masse with SrfI, diluted and self-ligated. These deleted plasmids would then be retransformed into host cells, resulting in a population of codon insertion mutants. These codon insertion mutants could then be used to study whatever function(s) are encoded in the target DNA biologically. The restriction site would again be very helpful for rapid mapping of the codon insertion. Other methods for codon insertion mutagenesis are taught in the art (33, 34).

4. "Carry along" transposition.

An artificial transposon carries a drug-selectable marker/or markers which allow selection of transposon-containing DNA target. The transposon also contains other DNA sequences adjacent to the marker (such as a gene). Hence, both the drug marker and the gene of interest are introduced upon integration of an artificial transposon with such a structure.

5. Fusion protein constructs.

An artificial transposon is designed such that, upon insertion into an open reading frame of a functional gene, a fusion protein would be produced. The fusion would be comprised of a portion of the original coding region of the functional gene, as well as a reporter which could be used to identify such active fusion proteins.

Example: An artificial transposon is created that contains the beta galactosidase gene. The reading frame is open from the terminus of the transposon through the beta galactosidase gene. Upon integration in a frame in a target gene, a fusion protein is produced that shows beta galactosidase activity.

6. Transgenic constructs.

A drug-selectable marker useful in the organism under study is introduced into a desired region of a gene or DNA within a cloning vector, for the ultimate purpose of introducing the segment of DNA into the host genome. This general approach has been reported for bacteria, yeast, drosophila, C. elegans, and mouse, as well as other mammals, and includes integrative knockouts such as those reported by M. Capecchi's lab.

Example 1: A researcher wishes to examine a 20 kb segment of mouse DNA for possible promoter activity both in cultured cells and in the context of the organism. An artificial transposon containing a reporter gene such as Chloramphenicol acetyl transferase (CAT), luciferase, or β-galactosidase could be integrated into the 20 kb region, and screened by restriction mapping. Next, the insertions could be tested for expression in cell culture or muscle injection transient assays. Finally, constructs showing expression could be used to generate transgenic animals. Such animals could be used to study the expression conferred by the promoter, by assaying reporter activity in various tissues or developmental states.

Example 2: An artificial transposon is created which contains a human transcriptional enhancer element that functions only in heart muscle tissue during early heart development. By inserting copies of this transposon in the upstream,

downstream, and intron regions of a gene of interest (cloned on a plasmid), constructs are generated where the gene would potentially be regulated by the enhancer in a tissue-specific and temporal manner. These constructs are used to generate transgenic animals where this gene would be expressed in this manner.

Example 3: Transgenic knockout constructs. An artificial transposon containing a NEO gene is created and integrated into a plasmid clone carrying the 5' portion of a gene of interest. The insertions are screened, and a single insertion occurring in the first exon of the gene, just downstream of the translation start codon AUG, is identified. The resulting construct is used directly to knockout the gene by generating a transgenic animal by ES technology. A second version would include the addition of a counterselectable marker at the 3' end of the construct to differentiate between homologous and non-homologous insertions. This counterselectable marker could be carried on a second artificial transposon. This general approach has been described by Capecchi and colleagues to generate "knockout mice" lacking the function of a particular gene.

Examples

Construction of pAT-1

pAT-1 (pSD544) and pAT-2 (pSD545) were constructed as follows. First, the plasmid pRS316 (ref. 15; a derivative of pBLUESCRIPT, Stratagene) was modified to eliminate the ampicillin resistance (amp^r) gene. This was accomplished by ligating together two fragments of pRS316 (a 2.1 kb *Ssp* I fragment and a 2.1 kb *Bsa* I/*Ssp* I fragment), thus creating the plasmid pSD528 which lacks a functional *bla* gene; this plasmid can be propagated in the pyrimidine-requiring *E. coli* strain MH1066 since the yeast *URA3* gene on this construct complements the bacterial *pyrF* auxotrophy (16). pAT-1 and pAT-2 were constructed from plasmid pSD528 by replacing the pBLUESCRIPT multicloning site (mcs) (from the unique *Kpn* I site to the unique *Sac* I site) with polymerase chain reaction (PCR) adapters containing the appropriate sequences to create the structure indicated in Fig. 2. These PCR adapters were generated using

primers SD112 (JB661) (5'- AAAA-GCTGGG-TACCGA-ACATGTT-CTCGAGGTCGACGGTATCG-3') and SD113 (JB662) (5'-GCGAATTGGA-GCTCGAAC-ATGTTCACCGC-GGTGG-CGGCCGCTC-3') with plasmids pBLUESCRIPT and pSD511 as templates. The resulting PCR products were digested with *Kpn* I and *Sac* I, and ligated to *Kpn I/Sac* I- digested pSD528 to generate pAT-1 and pAT-2. The structures of these constructs were confirmed by restriction mapping and sequence analysis.

***In vitro* reaction conditions.**

A typical *in vitro* DNA integration was carried out in a 20 μl reaction volume, and contained the following. 100-500 ng artificial transposon (0.8 kb), 1 μg CsCl-purified plasmid target (a 10 to 1 molar ratio of transposon to target), 2 μl 10 X reaction buffer (150 mM $MgCl_2$, 100 mM Tris HCl, pH 7.5, 100 mM KCl, and 10 mM DTT), 5 μl 20% [w/v] PEG 8000, 2 μl VLPs, and water to 20 μl. The reaction was incubated at 30° Celsius for 30 minutes followed by 37° Celsius for 10 minutes, and then was terminated by adding 1.0 μl 0.5 M EDTA and heating to 65° Celsius for 20 minutes. Finally, the nucleic acids were phenol/chloroform extracted, ethanol precipitated, collected by centrifugation, washed with 70% ethanol, and resuspended in 10 μl TE (10 mM Tris, pH 8.0, 1 mM EDTA). 1 μl was used to transform 6 μl DH10B *E. coli* (Gibco/BRL) to drug resistance by electroporation.

PCR, sequencing, primers, plasmid constructions, CsCl preps.

The PCR was carried out using reagents obtained from Perkin Elmer, as described (17). DNA sequencing was carried out using Sequenase (USB), and analyzed as described (18). Custom oligonucleotide primers were obtained from Operon Technologies, Inc. (Alameda, California). The two primers used for sequencing from within the PART were SD111 (JB563) (5'-GACACTCTGTTA-TTACAAATCG-3') and SD110 (JB532) (5'-GGTGATCCCTGAGCAGGTGG-3'). The integration site of each PART insertion was determined using either one or

both of these primers, and analyzed with the aid of the Wisconsin GCG package. Plasmids were constructed using standard DNA cloning methods (19), and were purified from *E.coli* cultures by either STET miniprep (20) or alkaline lysis followed by CsCl banding (21).

Preparation of artificial transposons from pAT-1 and derivatives.

20μg of CsCl-purified plasmid DNA was digested with 50 units of *Xmn* I (Boehringer Mannhiem) for 4 hours at 37° Celsius. The resulting fragments were separated on a 1% agarose/TBE gel, and the transposon fragment was electroeluted from the gel using an IBI electroelution device.

Recovery of clones carrying transposon insertions using ampicillin/trimethoprim plates.

E. coli clones carrying plasmids with transposon insertions were identified by selection on M9 minimal plates (22) containing 1.0 mM thiamine HCl, 50 μg/ml ampicillin (Amp) and 100 μg/ml trimethoprim (Tri; Sigma). After one to two days incubation at 37° Celsius, the majority of colonies growing on M9/Amp/Tri plates contained plasmids with a transposon insertion. Dilutions of the transformation were routinely plated on LB plates containing 50 μg/ml Amp (22); this control monitored the number of target plasmids successfully carried through the procedure. When compared to the number of colonies on M9/Amp/Tri plates, the frequency of transposon insertion could be estimated (frequency of insertion = [# colonies on M9/Amp/Tri plates] / [# colonies on LB/Amp plates]). A positive control plasmid, pSD511, containing both Amp^R and Tri^R markers, routinely gave rise to equivalent numbers of colonies on LB/Amp (50 ug/ml), M9/Tri (100 ug/ml), or M9/Amp/Tri (50/100ug/ml) plates under these conditions.

Transformation of *E. coli*.

The two strains transformed routinely in this work were DH5α (23) and DH10B (24). DH5α was prepared for electroporation as described (25), and electrocompetent DH10B cells were purchased from Gibco/BRL. Transformation by electroporation was accomplished for both strains using a Biorad Genepulser with 1 mm cuvettes and the following settings: capacitance: 25 μFD; voltage: 1.8 kV; and resistance: 200 ohms. Using pUC19 or pBLUESCRIPT as a test plasmid, freshly-prepared electrocompetent DH5α generally showed transformation efficiencies of 10^7 - 10^8 colonies/μg DNA, whereas electrocompetent DH10B purchased from BRL/Gibco generally showed efficiencies of 5 X 10^8 to 5 X 10^9 colonies/μg DNA.

VLP preparation.

VLPs were prepared from yeast cultures as described (26). Fractions from the final sucrose gradient containing integrase activity were aliquoted and frozen at -70° Celsius where they were stable for more than 6 months.

***In vitro* integration of "primer island" transposons into a cloned segment of yeast chromosome III carried on a plasmid target.**

We next generated PART insertions *in vitro* using various plasmid targets. One of the primary test clones consisted of a pRS200 backbone (a derivative of pBLUESCRIPT) with an 8.0 kb insert that spans bp 136,155 to 144,333 of yeast chromosome III; this plasmid is called p76-2. With a single *in vitro* integration reaction, we recovered approximately 13,000 PART insertions in p76-2 (Table 1).

TABLE 1

Table 1. Recovery of PART insertions into clone 76-2.

Rxn	EDTA[a]	Total transformants[b]	Total insertion plasmids[c]	Frequency of transposition[d]
1.	-	0	0	-
2.	-	3.1×10^8	4.5×10^8	-
3.	-	3.1×10^8	1.3×10^4	4.2×10^{-5}
4.	+	5.7×10^8	5.0×10^2	9.1×10^{-7}

Reaction 1) negative transformation control (no DNA added); 2) positive transformation control (pSD511, which contains both Amp^R and Tri^R markers); 3) complete integration reaction using p76-2 as the target; 4) same as reaction 3, but EDTA was added (inhibits integrase activity).

a. +, EDTA added to 25 mM
b. Total number of Amp^R transformants
c. Total number of Amp^R/Tri^R transformants
d. Number of transpositions into target plasmid (Amp^R/Tri^R colonies) divided by the total number of transformants (Amp^R colonies)

By measuring the number of colonies transformed to ampicillin resistance vs. combined trimethoprim and ampicillin resistance, we determined that the frequency of transposon insertion recovery was approximately 4.2 X 10^{-5} (i.e., 1 insertion per 2.4 X 10^{4} target molecules; Table 1). Although this frequency is not likely to represent the upper limits of optimization, it is sufficiently high that a large number of insertion events are readily recovered, while sufficiently low that a single target is generally limited to a single transposon insertion (two transposon insertions in a single target might be useful for some purposes, but would render the molecule useless as a sequencing template).

Analysis of 156 randomly chosen Amp^{R}/Tri^{R} colonies indicated that PART insertions occurred into all areas of the plasmid target, including both the pRS200 backbone (6.0 kb) and the 8.0 kb chromosome III insert, as determined by restriction mapping and/or sequence analysis (Table 2).

TABLE 2

Table 2. Examination of Tri^R/Amp^R colonies from a single *in vitro* integration reaction.

		%
Total number of Tri^R clones examined	156	100
# minipreps recovered	153	98
# easily-identifiable insertions	134	86
In insert	78	50
In vector	56	36
Other	19	12
double insertions/cotransformants[a]	13	8
unknown plasmid map	5	3
no transposon	1	< 1

a. This class contains some plasmids that apparently had two independent insertions in the target as determined by restriction mapping, and others with DNA sequence that was readable to the insertion junction, at which point two superimposed sequences were observed.

More than 86% of these 156 clones (134) had easily-identifiable PART insertions; of these, 78 (50%) were in the cloned 8 kb insert, while 56 (36%) were in the vector. A small percentage of the clones were found to have two superimposed restriction maps/and or sequences. There are several likely explanations for this result, including the possibility that two plasmids transformed a single *E. coli* clone, or that two transposon insertions occurred into a single plasmid target; the available evidence indicates that most of these clones are explained by such mechanisms. Hence, a small portion of clones recovered from an *in vitro* integration reaction would not be suitable for direct DNA sequence analysis for this reason (12 % in this example, Table 2). Likewise, vector insertions would not be useful for sequencing the insert. Nevertheless, one of every two Amp^R/Tri^R colonies analyzed from this single reaction could be used directly to obtain DNA sequence from the cloned insert. Furthermore, analysis of only 156 minipreps led to the assembly of 78 useful insertions in an 8 kb insert, corresponding to an expected distribution of roughly one insertion per 100 bp.

The distribution of individual insertions of the artificial transposon relative to adjacent insertions is shown in Table 3.

TABLE 3

Tabulation of PART insertion data from plasmid target p76-2

Insertion Plasmid	Insertion point in p76-2 (chr III numbering)	distance to 5-prime clone
5-prime end	136155	-
151	136394 R	239
72	136397 F	3
25	136415 R	18
116	136425 R	10
107	136460 R	35
93	136576 R	16
155	136611 F	35
135	136685 F	74
46	136724 R	39
141	136767 F	43
84	136832 R	65
33	137058 F	226
70	137165 F	107
124	137192 R	27
101	137347 R	155
59	137451 F	104
17	137622 R	171
77	137657 F	35
89	137811 F	154
147	137879 R	68
54	138127 R	248
145	138161 F	34
105	138175 F	14
16	138263 R	88
146	138345 F	82
20	138503 F	158
122	138581 R	78
63	138587 F	6
125	138588 F	1
86	138618 R	30
152	138702 F	84
110	138720 F	18
32	138747 R	27
117	138771 F	24
114	138819 R	48
94	138905 R	86
40	138906 R	1
112	139283 R	377
41	139291 R	8
119	139332 R	41
102	139529 F	197
19	139551 R	22
134	139690 R	139

85	139863 R	173
42	139980 R	117
22	140052 R	72
73	140176 R	124
80	140259 R	83
38	140360 F	101
90	140446 R	86
103	140794 R	348
24	141023 R	229
57	141024 R	1
2	141074 R	50
49	141174 F	100
11	141412 F	238
68	141633 F	221
58	141765 F	132
12	141770 R	5
142	141836 R	66
29	141876 F	40
69	142015 R	139
31	142027 R	12
4	142094 R	67
78	142180 F	86
60	142226 R	46
127	142382 R	156
3	142551 R	169
74	142713 F	162
108	142820 F	107
6	143141 F	321
109	143165 R	24
149	143333 R	168
27	143616 F	283
39	143856 F	240
51	143921 F	65
13	144076 F	155
66	144127 F	51
3-prime end	144333	206

Statistics on insertions

n = 78
Mean interval distance = 102.3 +/- 88.1
Insertions/kb for each 1 kb of target:

Region of target	Number of insertions per kb target DNA
136,155 to 137,000	13
137,000 to 138,000	9
138,000 to 139,000	17
139,000 to 140,000	14
140,000 to 141,000	6
141,000 to 142,000	10
142,000 to 143,000	9
143,000 to 144,000	6
144,000 to 144,333	6

Mean number of insertions per kb target DNA = 10.2 +/- 3.7
Orientation
Forward 34 (44%)
Reverse 44 (56%)

Since the entire yeast chromosome III sequence has been previously determined (27), we could easily identify the precise sites of transposon integration by determining the nucleotide sequences at the insertion junctions. Indeed, the 78 PART insertions were found to be distributed throughout the entire 8 kb insert (Fig. 3). A little less than half of these insertions were in the forward orientation (34/78 or 44%), indicating a slight orientation bias for this target. However, since primer extensions can be initiated into the sequences flanking the insertion on both sides irrespective of the PART orientation, an orientation bias does not affect the utility of the PART insertion for purposes of DNA sequencing. The mean distance between adjacent insertions was 102.3 +/- 88.1 overall. Only six of the intervals were greater than 250 bp, and the largest of these was only 377 bp. Hence, the vast majority of the intervals between adjacent transposon insertions were well below the maximum distance that can be reached with an average primer extension under sequencing conditions. A property of Ty1 integrase is that it creates characteristic 5 bp target sequence duplications flanking the insertion site upon integration (10-12, 28). As expected, 5 bp target site duplications were found at each PART integration site examined (only a small portion of the insertions were sequenced at both ends in this example). No deletions or rearrangements were observed.

A conceptual primer extension contig map based on our results is shown in Fig. 4. We have made the assumption that each primer extension would lead to the successful recovery of 250 bp of useful sequence information. 100% of the sequence would be recovered on one strand or the other using the 78 PART insertions shown in Fig. 3. Only 6 gaps (3 on the top strand, and 3 on the bottom; each < 150 bp) would exist. But because the two initial primer extensions flanking such a gap would cross in the middle on opposite strands, uninterrupted DNA sequence would be recovered on one strand or the other. Nevertheless, the gaps on the remaining strand could be closed with either: i) additional PART insertions in the necessary regions, identified with appropriate restriction mapping, ii) custom primers, or iii) longer sequencing runs. Of course, we have made the

assumption that only 250 bp of sequence information can be recovered from a single primer extension; in fact, greater than 400 is routinely obtained with automated sequencers, and 800 to 1000 is becoming possible with automated sequencers in development. Hence, if the mean readable sequence is extended to 400 bp, 100% of the sequence could be easily recovered using fewer than 78 PART insertions.

Other targets tested.

In addition to clone 76-2 containing a DNA insert from yeast chromosome III, we have tested other plasmid targets. These plasmids had a variety of backbone structures and carried various cloned inserts (Table 3). The backbones included pUC19 and pBLUESCRIPT as well as others, and the DNA inserts originated from different species including yeast and human. In each case, results similar to those shown for clone 76-2 were obtained: i) insertions were mapped to all regions of these targets, ii) a large number of insertions was readily recovered from reactions using each target, and iii) recovered insertions consistently served as successful sequencing templates. Moreover, in two cases other than p76-2 (pCAR143 and pWAF-1; table 3), this system was used to recover 90-100% of the nucleotide sequence from clones with previously unknown sequences. Hence, *in vitro* integration of artificial transposons is expected to work well with most or all plasmid targets, making it both a generally useful sequencing tool and a general method of integrating new DNA sequences into plasmid targets to generate recombinant DNA molecules.

MAPPING AND SEQUENCING COSMID DNA USING ARTIFICIAL TRANSPOSONS.

We have demonstrated that artificial transposons can be efficiently integrated into a wide variety of plasmid targets *in vitro* using Ty1 integrase. Our data indicate that cosmids can also serve as targets for integration using the same protocol as that used for plasmids. Hence, DNA mapping, sequencing and functional genetic analysis can be performed directly on large (30-50 kb) DNA

inserts propagated in cosmid cloning vectors. These results confirm that the target for artificial transposon insertion is flexible; in principle, any DNA molecule could serve as a target for the integration of artificial transposons *in vitro*. The resulting recombinant could be used either to analyze the regions surrounding the insertion, or for any other purpose generally provided by recombinant DNA molecules, including but not limited to functional genetic analysis and recombinant DNA engineering.

Supportive Data.

1. AT-2 insertions have been generated in four different cosmids using the same methods used to generate AT-2 insertions in plasmids. These include three cosmids obtained from the Lawrence Livermore Genome Center, F23932, F13544, and F20080, each consisting of a Lawrist cloning vector and an insert of approximately 30 to 50 kb derived from Human chromosome 19, as well as one additional cosmid, JEDI-C, also carrying an insert of approximately 30 to 50 kb.

2. Restriction mapping. Insertions mapped to all regions of target cosmids supporting a quasi-random model for integration as was observed for plasmids.

3. AT-2/cosmid recombinants were successfully used as sequencing templates with ABI Prism cycle sequencing technology. More than 100 cosmid recombinants (including 17 from a previously uncharacterized cosmid, F23932) have been evaluated as sequencing templates and the majority (>90%) gave readable sequence of 300 to 600 bp for each primer extension with high levels of accuracy (>95%).

4. 8 kb of previously characterized sequence of the cosmid F13544 was re-analyzed with AT-2 insertions and Prism sequencing technology (see Figure 11). All available data indicate that this method is fully capable of recovering accurate sequence information comparable with other state-of-the-art methods.

Thus, cosmids can be analyzed with artificial transposon technology at both the structural and sequence levels. It is predicted that cosmid recombinants could also be used for functional genetic analysis. The advantages of direct analysis of DNA inserts propagated as large recombinant cosmid molecules are as follows. 1)

Direct analysis allows the original linkage of the insert to be maintained throughout the analysis, avoiding the problems associated with destroying linkage, e.g. as in shotgun sequencing, 2) direct analysis allows the navigation of "difficult" DNA inserts containing complex repeat structures and 3) map and sequence information from a single transposon insertion can be used in concert to permit simplified sequence assembly schemes.

VARYING THE NUCLEOTIDE SEQUENCE AND STRUCTURE OF THE ARTIFICIAL TRANSPOSON.

Our initial experiments were performed with the artificial transposon AT-2. Our results suggested that the sequence and design of the artificial transposon were likely to be flexible. We have now tested this hypothesis by designing and constructing artificial transposons with a variety of sequences and features. Like AT-2, these artificial transposons were constructed in pAT-1 or pAT-2 vectors or derivatives (Figure 12), relying upon the same multicloning site for construction of these plasmids, and the same Xmn I restriction strategy for preparation of the transposon from the vector (in each case, the artificial transposon bears the same relationship to its parent plasmid that AT-2 bears to pAT-2). The results of our studies indicate that, indeed, the sequence of the artificial transposon can be varied substantially while retaining transposition activity. Thus, any desired feature can, in principle, be incorporated into an artificial transposon using methods available for engineering plasmids or linear DNA molecules. The following artificial transposons have been constructed and, where indicated, have also been tested for transposition or otherwise.

1. AT-2. The artificial transposon AT-2 contains at its termini 4 bp of Ty1 U3 terminal sequences (5'-AACA-3'); subterminal primer sites SD110, 111, 118 and 119 used for PCR or sequencing; subterminal restriction sites for mapping and engineering; a drug-selectable dhfr cassette conferring resistance to the antibiotic trimethoprim in E. coli. AT-2 was constructed in the plasmid pAT-2.

2. AT-2-TRP1 This transposon is identical to AT-2 with the exception that the yeast auxotrophic marker TRP1 has been added at the unique Hind III site

present in pAT-2. The overall transposon is approximately 1.6 kb in length. The TRP1 marker is selectable in both bacteria and yeast. AT-2-TRP1 transposes *in vitro* using the methods established for AT-2. Insertions were found to be quasi-randomly distributed. Following integration into plasmid targets and transformation into yeast, the locations of functionally active regions on the target plasmid were mapped by insertional inactivation. For example, in one plasmid target containing the yeast URA3 and LYS2 genes (pSD553), AT-2-TRP1 insertions were found to inactivate these genes upon insertion within their open reading frames, leading to a Ura- or Lys- phenotype in yeast (Table 4). When insertions occurred outside of these genes in the same target, however, the plasmids were still capable of yielding a Ura+, Lys+ phenotype in yeast. In all cases, a Trp+ phenotype due to the TRP1 marker on the transposon was observed in yeast.

Table 4

AT2-TRP1 /pSD553 recombinants

	Phenotypes					
Recombinant	Amp	Tmp	Ura	Lys	Trp	AT2-TRP1 ins. site
1	R	R	-	+	+	URA3 ORF
2	R	R	+	+	+	LYS2 3' UTR
3	R	R	+	-	+	LYS2 ORF
4	R	R	+	+	+	vector (between Amp and CEN)
5	R	R	+	-	+	ND
6	R	R	+	-	+	LYS2 ORF
7	R	R	+	-	+	ND
8	R	R	+	-	+	ND
9	R	R	+	+	+	ND
10	R	R	+	+	+	ND
11	R	R	+	+	+	ND
12	R	R	-	+	+	URA3 ORF
13	R	R	-	+	+	ND
14	R	R	+	-	+	ND
15	R	R	+	-	+	ND
16	R	R	+	+	+	ND
17	R	R	+	-	+	ND
18	R	R	+	+	+	ND
19	R	R	+	+	+	ND
20	R	R	+	-	+	ND
553 +C	R	S	+	+	-	
554 +C	R	S	-	-	+	

Legend. Results of functional analysis of pSD553 recombinants in yeast.
The results of functional analysis of 20 independent AT-2-TRP1 recombinants of pSD553 are tabulated. The recombinants were first generated *in vitro*, and recovered in E. coli by selection for trimethoprim resistance. After mapping sites of insertion, each recombinant was transformed into the yeast strain yPH499 (ura352, lys801, trp1D63) and plated on synthetic media lacking uracil, lysine, or tryptophan. Finally, transformants were replica plated to each media and their phenotype scored. R = resistant; S = sensitive; + = growth on media lacking the specified nutrient; - = no growth. The sites of six insertion events determined by sequence analysis.are indicated in the last column.

3. AT-2-LacZ. This transposon is identical to AT-2 with the exception that the LacZ marker has been inserted between the unique Sal I and Xho I sites of pAT-2. The overall transposon is approximately 4 kb in length. AT-2-LacZ transposes *in vitro* with the methods established for AT-2. When insertion occurs in-frame with an open reading frame present on the target, the resulting recombinant encodes a fusion protein which can be assayed for function in the appropriate host using an indicator substrate such as X-gal. We have tested this approach on an 8 kb segment of yeast chromosome III, and AT-2-LacZ accurately predicted the location of a known gene present on the clone. Thus, artificial transposons can be used to functionally map the location of genes by making reporter fusion proteins.

4. AT-2-neo. This transposon is identical to AT-2 with the exception of the addition of a neo cassette at the unique Hind III site in pAT-2. This transposon has not been tested functionally.

5. AT-3. This transposon was derived from pAT-1 by adding a cassette encoding the neo gene at the unique Bam HI site of pAT-1. This neo cassette confers resistance to G418 in yeast and kanamycin in bacteria. AT-3 transposes *in vitro* with methods established for AT-2. The orientation of the neo cassette is left to right, with the unique Not I site of AT-3 on the left, and the unique Xho I site on the right, of the cassette.

6. AT-4. This transposon is identical to AT-3 with the exception that the neo cassette is in the opposite orientation. AT-4 transposes *in vitro* with the methods established for AT-3.

7. AT-5. This transposon was designed to contain the bla (ampicillin resistance) gene and is otherwise identical to AT-3. AT-5 has been designed but not built nor tested.

These results collectively indicate that the cis sequences of the artificial transposon can be varied extensively while retaining transposition function and quasi-random integration *in vitro*. Thus, transposons with custom features can be constructed and used for a variety of purposes. These features include both

functional and non-functional DNA sequences, primer sites, restriction sites, and otherwise useful sequences.

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SEQUENCE LISTING

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(ii) TITLE OF INVENTION: In Vitro Transposition of Artificial Transposons

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(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4164 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: pAT-1

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4933 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: pAT-2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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GTAAGACACG ACTTATCGCC ACTGGCAGCA GCCACTGGTA ACAGGATTAG CAGAGCGAGG 3720

TATGTAGGCG GTGCTACAGA GTTCTTGAAG TGGTGGCCTA ACTACGGCTA CACTAGAAGG 3780

ACAGTATTTG GTATCTGCGC TCTGCTGAAG CCAGTTACCT TCGGAAAAAG AGTTGGTAGC 3840

TCTTGATCCG GCAAACAAAC CACCGCTGGT AGCGGTGGTT TTTTTGTTTG CAAGCAGCAG 3900

ATTACGCGCA GAAAAAAAGG ATCTCAAGAA GATCCTTTGA TCTTTTCTAC GGGGTCTGAC 3960

GCTCAGTGGA ACGAAAACTC ACGTTAAGGG ATTTTGGTCA TGAGATTATC AAAAAGGATC 4020

TTCACCTAGA TCCTTTTAAA TTAAAAATGA AGTTTTAAAT CAATCTAAAG TATATATGAG 4080

TAAACTTGGT CTGACAGTTA CCAATGCTTA ATCAGTGAGG CACCTATCTC AGCGATCTGT 4140

CTATTTCGTT CATCCATAGT TGCCTGACTC CCCGTCGTGT AGATAACTAC GATACGGGAG 4200

GGCTTACCAT CTGGCCCCAG TGCTGCAATG ATACCGATTA TTGAAGCATT TATCAGGGTT 4260

ATTGTCTCAT GAGCGGATAC ATATTTGAAT GTATTTAGAA AAATAAACAA ATAGGGGTTC 4320

CGCGCACATT TCCCCGAAAA GTGCCACCTG GGTCCTTTTC ATCACGTGCT ATAAAAATAA 4380

TTATAATTTA AATTTTTTAA TATAAATATA TAAATTAAAA ATAGAAAGTA AAAAAAGAAA 4440

TTAAAGAAAA AATAGTTTTT GTTTTCCGAA GATGTAAAAG ACTCTAGGGG GATCGCCAAC 4500

AAATACTACC TTTTATCTTG CTCTTCCTGC TCTCAGGTAT TAATGCCGAA TTGTTTCATC 4560

TTGTCTGTGT AGAAGACCAC ACACGAAAAT CCTGTGATTT TACATTTTAC TTATCGTTAA 4620

TCGAATGTAT ATCTATTTAA TCTGCTTTTC TTGTCTAATA AATATATATG TAAAGTACGC 4680

TTTTTGTTGA AATTTTTTAA ACCTTTGTTT ATTTTTTTTT CTTCATTCCG TAACTCTTCT 4740

ACCTTCTTTA TTTACTTTCT AAAATCCAAA TACAAAACAT AAAAATAAAT AAACACAGAG 4800

TAAATTCCCA AATTATTCCA TCATTAAAAG ATACGAGGCG CGTGTAAGTT ACAGGCAAGC 4860

GATCCGTCCT AAGAAACCAT TATTATCATG ACATTAACCT ATAAAAATAG GCGTATCACG 4920

AGGCCCTTTC GTC 4933

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 864 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: PART

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGTTCACCGC GGTGGCGGCC GCTCTAGAAC TAGTGGATCC TGCAAGCAGG ATAGACGGCA 60

TGCACGATTT GTAATAACAG AGTGTCTTGT ATTTTTAAAG AAAGTCTATT TAATACAAGT 120

GATTATATTA ATTAACGGTA AGCATCAGCG GGTGACAAAA CGAGCATGCT TACTAATAAA 180

ATGTTAACCT CTGAGGAAGA ATTGTGAAAC TATCACTAAT GGTAGCTATA TCGAAGAATG 240

GAGTTATCGG GAATGGCCCT GATATTCCAT GGAGTGCCAA AGGTGAACAG CTCCTGTTTA 300

AAGCTATTAC CTATAACCAA TGGCTGTTGG TTGGACGCAA GACTTTTGAA TCAATGGGAG 360

CATTACCCAA CCGAAAGTAT GCGGTCGTAA CACGTTCAAG TTTTACATCT GACAATGAGA 420

ACGTATTGAT CTTTCCATCA ATTAAAGATG CTTTAACCAA CCTAAAGAAA ATAACGGATC 480

ATGTCATTGT TTCAGGTGGT GGGGAGATAT ACAAAAGCCT GATCGATCAA GTAGATACAC 540

TACATATATC TACAATAGAC ATCGAGCCGG AAGGTGATGT TTACTTTCCT GAAATCCCCA 600

GCAATTTTAG GCCAGTTTTT ACCCAAGACT TCGCCTCTAA CATAAATTAT AGTTACCAAA 660

TCTGGCAAAA GGGTTAACAA GTGGCAGCAA CGGATTCGCA AACCTGTCAC GCCTTTTGTG 720

CCAAAAGCCG CGCCAGGTTT GCGATCCGCT GTGCCAGGCG TTAGGCGTCA TATGAAGATT 780

TCGGTGATCC CTGAGCAGGT GGCGGAAACA TTGGATGCTG AGAATTCGAT ATCAAGCTTA 840

TCGATACCGT CGACCTCGAG AACA 864

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: JB563

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GACACTCTGT TATTACAAAT CG 22

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: JB532

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGTGATCCCT GAGCAGGTGG 20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: JB661

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AAAAGCTGGG TACCGAACAT GTTCTCGAGG TCGACGGTAT CG 42

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: JB662

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCGAATTGGA GCTCGAACAT GTTCACCGCG GTGGCGGCCG CTC 43

CLAIMS

1. A method for providing templates for DNA sequencing, comprising the steps of:

incubating *in vitro*: (1) a population of a target DNA, said target DNA comprising a region of DNA to be sequenced, (2) a retroviral or retrotransposon integrase, and (3) an artificial transposon having two termini which are substrates for said integrase, wherein the molar ratio of artificial transposon to target DNA is at least 1:1, to form a population of target DNAs with quasi-randomly integrated insertions of the artificial transposon;

transforming host cells with the population of target DNAs with quasi-randomly integrated insertions of the artificial transposon;

selecting those host cells which have been transformed with a target DNA with an insertion of the artificial transposon;

isolating target DNA with an insertion of the artificial transposon from those host cells which have been transformed with a target DNA with an insertion of the artificial transposon, said target DNA with an insertion of the artificial transposon being suitable for use as a DNA sequencing template.

2. The method of claim 1 wherein said integrase is yeast retrotransposon Ty1 integrase.

3. The method of claim 1 wherein said target DNA is a plasmid.

4. The method of claim 1 wherein said target DNA is a cosmid.

5. The method of claim 2 wherein said integrase is supplied as Ty1 virus-like particles.

6. The method of claim 2 wherein each of said termini contains Ty1 U3 sequences.

7. The method of claim 6 wherein said termini consist of 4 to 11 base pairs.

8. The method of claim 1 wherein said artificial transposon is provided by restriction digestion with an enzyme which generates blunt ends.

9. The method of claim 8 wherein said restriction enzyme is *Xmn*I.

10. The method of claim 1 wherein said step of transforming is facilitated by electroporation.

11. The method of claim 1 wherein said molar ratio is at least 2.5:1.

12. A method for sequencing DNA, comprising the steps of:

incubating *in vitro* (1) a population of a target DNA, said target DNA comprising a region of DNA to be sequenced, (2) a retrovirus or retrotransposon integrase, and (3) an artificial transposon having two termini which are substrates for said integrase, wherein the molar ratio of artificial transposon to target DNA is at least 1:1, to form a population of target DNAs with quasi-randomly integrated insertions of the artificial transposon;

transforming host cells with the population of target DNAs with quasi-randomly integrated insertions of the artificial transposon;

selecting those host cells which have been transformed with a target DNA with an insertion of the artificial transposon;

isolating target DNA with an insertion of the artificial transposon from those host cells which have been transformed with a target DNA with an insertion of the artificial transposon, said target DNA with an insertion of the artificial transposon being suitable for use as a DNA sequencing template;

hybridizing to said isolated target DNA with an insertion of the artificial transposon a primer which is complementary to a terminus of the artificial transposon;

extending said primer to determine a nucleotide sequence of DNA flanking said artificial transposon in said isolated target DNA with an insertion of the artificial transposon.

13. The method of claim 12 wherein said integrase is yeast retrotransposon Ty1 integrase.

14. The method of claim 12 wherein said target DNA is a plasmid.

15. The method of claim 12 wherein said target DNA is a cosmid.

16. The method of claim 13 wherein said integrase is supplied as Ty1 virus-like particles.

17. The method of claim 16 wherein each of said termini is derived from a Ty1 U3 sequence.

18. The method of claim 17 wherein said termini consist of 4 to 11 base pairs.

19. The method of claim 12 wherein said artificial transposon is probided by restriction digestion with an enzyme which generates blunt ends.

20. The method of claim 19 wherein said restriction enzyme is *Xmn* I.

21. The method of claim 12 wherein said molar ratio is at least 2.5:1.

22. The method of claim 12 wherein said step of transforming is facilitated by electroporation.

23. A method for sequencing DNA, comprising the steps of:

providing a population of target DNAs with quasi-randomly integrated insertions of an artificial transposon, said artificial transposon having termini which are substrates for a retrovirus or a retrotransposon, said population of target DNAs having been formed by *in vitro* insertion of said artificial transposon into the target DNAs using a retroviral or retrotransposon integrase and a molar ratio of artificial transposon to target DNA of at least 1:1;

hybridizing to individual target DNAs of said population a primer which is complementary to a terminus of the artificial transposon;

extending said primer to determine a nucleotide sequence of target DNA flanking said artificial transposon.

24. The method of claim 23 wherein the integrase is yeast retrotransposon Ty1 integrase.

25. The method of claim 23 wherein said target DNA is a plasmid.

26. The method of claim 23 wherein said target DNA is a cosmid.

27. The method of claim 24 wherein said integrase is supplied as Ty1 virus-like particles.

28. The method of claim 24 wherein each of said termini is derived from a Ty1 U3 sequence.

29. The method of claim 28 wherein said termini consist of 4 to 11 base pairs.

30. The method of claim 23 wherein said molar ratio is at least 2.5:1.

31. A kit for DNA sequencing, comprising:

an artificial transposon having termini which are substrates for a retroviral or retrotransposon integrase;

a retroviral or retrotransposon integrase;

a buffer for *in vitro* transposition of said artificial transposon, said buffer having a pH of 6 to 8 and 1 to 50 mM of a divalent cation; and

a primer which is complementary to a terminus of said artificial transposon.

32. The kit of claim 31 wherein said integrase is yeast retrotransposon Ty1 integrase.

33. The kit of claim 32 wherein said integrase is supplied as Ty1 virus-like particles.

34. The kit of claim 32 wherein said artificial transposon is isolated by digestion with a restriction enzyme which creates blunt ends.

35. The kit of claim 34 wherein said restriction enzyme is *Xmn* I.

36. An artificial transposon consisting of an isolated, linear, blunt-ended DNA molecule comprising:

a marker DNA;

a sequence of yeast retrotransposon Ty1, said sequence selected from the group consisting of a U5 sequence and a U3 sequence, said sequence being upstream and flanking said marker gene, said sequence consisting of 4 to 11 bp of terminal sequences of said Ty1; and

a sequence of yeast retrotransposon Ty1, said sequence selected from the group consisting of a U5 sequence and a U3 sequence, said sequence being downstream and flanking said marker gene, said sequence consisting of 4 to 11 bp of terminal sequences of said Ty1, wherein each of said sequences of yeast retrotranspson Ty1 are at the termini of said linear DNA molecule.

37. The artificial transposon of claim 36 which is isolated by digestion of a DNA molecule containing said artificial transposon with a restriction enzyme which creates blunt ends when it cleaves DNA.

38. The artificial transposon of claim 37 wherein said restriction enzyme is *Xmn* I.

39. The artificial transposon of claim 36 wherein the marker DNA is an antibiotic resistance determinant.

40. The artificial transposon of claim 36 wherein the marker DNA is a dihydrofolate reductase gene (*dhfr*).

41. The artificial transposon of claim 36 wherein the marker DNA is a yeast auxotrophic marker.

42. The artificial transposon of claim 36 wherein each of the sequences flanking the marker DNA consist of the sequence 5'-AACA-3'.

43. The artificial transposon of claim 36 wherein each of the sequences flanking the marker gene are derived from U3 sequences.

44. A DNA molecule useful for generating artificial transposons, comprising:

an origin of replication;

a first selectable marker DNA;

two blunt-ended transposon termini of at least 4 bp each, said termini being substrates for yeast retrotransposon Ty1 integrase, said transposon termini flanking a first restriction enzyme site useful for insertion of a second selectable marker gene to form an artificial transposon;

a second restriction enzyme site flanking said two transposon termini, wherein digestion with said second restriction enzyme liberates a blunt-ended fragment having said transposon termini at either end of the fragment, the fragment thereby liberated being an artificial transposon.

45. A method for *in vitro* generation of insertions into a target DNA, comprising the steps of:

incubating *in vitro* (1) a population of a target DNA, (2) a retroviral or retrotransposon integrase, and (3) an artificial transposon having termini which are substrates for said integrase, wherein the molar ratio of artificial transposon to target DNA is at least 1:1, to form a population of target DNAs with quasi-randomly integrated insertions of the artificial transposon;

transforming a host cell with the population of target DNAs with quasi-randomly integrated insertions of the artificial transposon;

selecting those host cells which have been transformed with a target DNA with an insertion of the artificial transposon.

46. The method of claim 46 wherein the molar ratio of artificial transposon to target DNA is at least 2.5:1.

FIG. 1

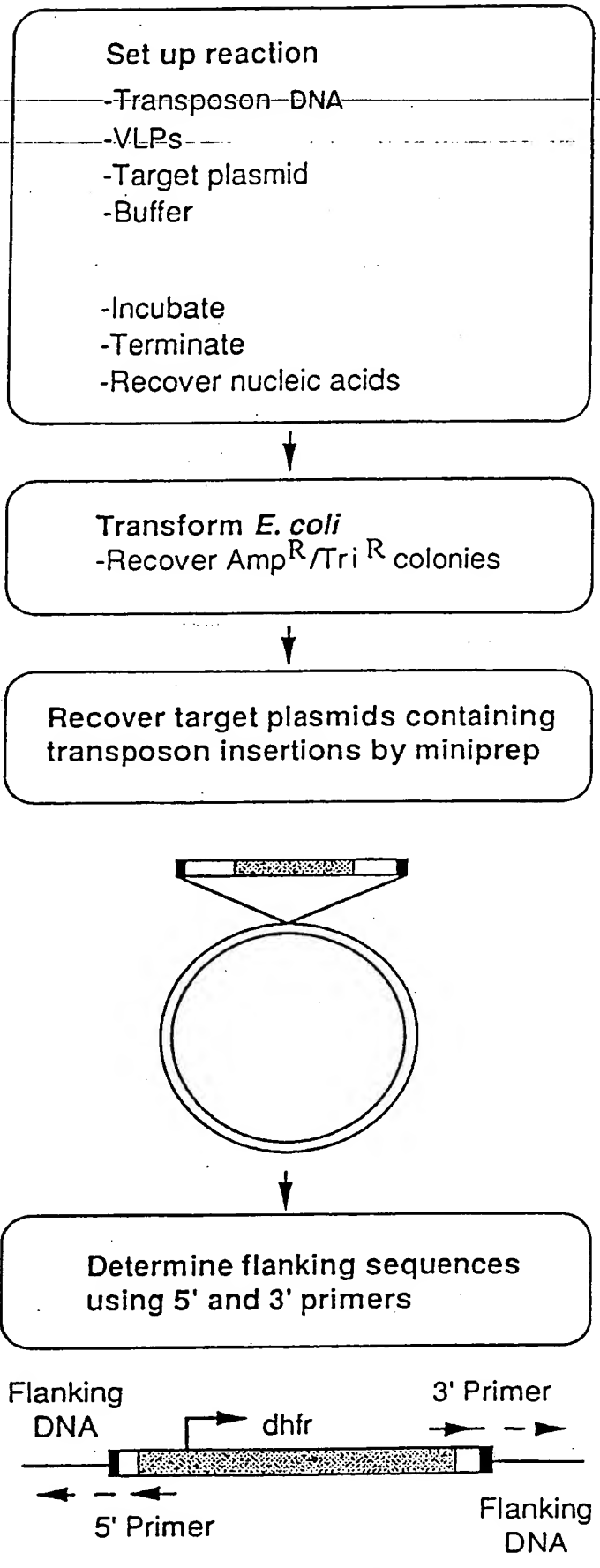

Set up reaction
-Transposon DNA
-VLPs
-Target plasmid
-Buffer
-Incubate
-Terminate
-Recover nucleic acids
Transform E. coli
-Recover Amp^R/Tri^R colonies
Recover target plasmids containing transposon insertions by miniprep
Determine flanking sequences using 5' and 3' primers
Flanking DNA
dhfr
3' Primer
5' Primer
Flanking DNA

FIG. 2A

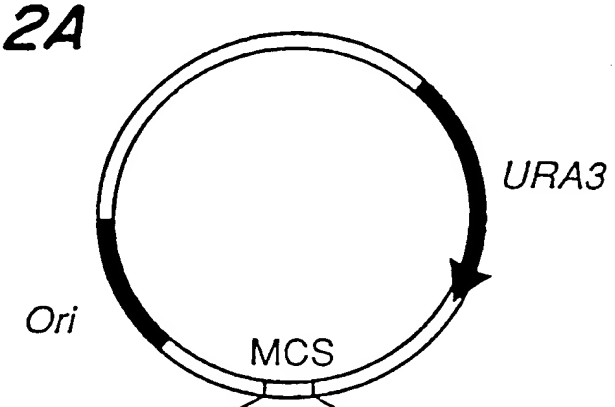


FIG. 2B

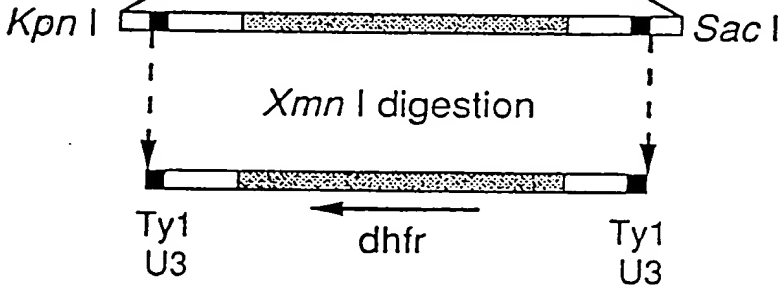


FIG. 2C

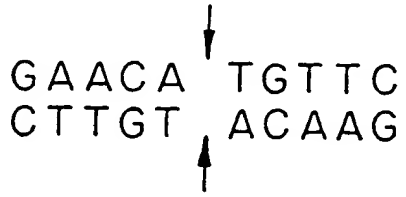
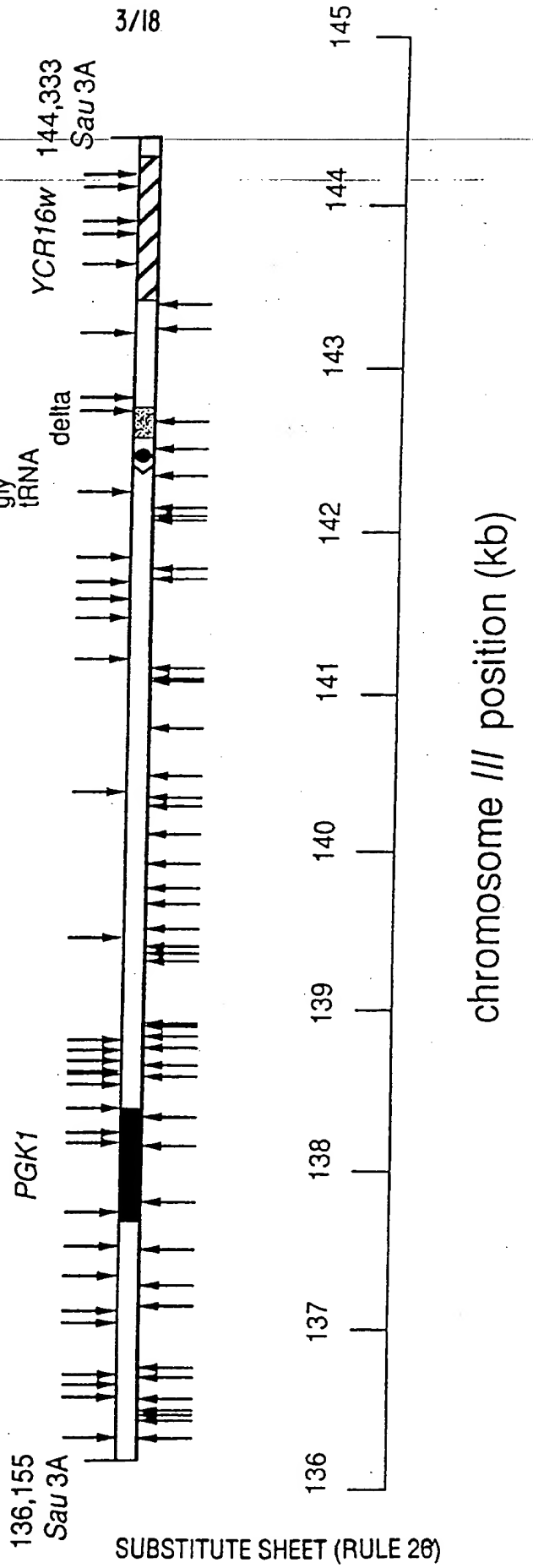


FIG. 3


144,333
Sau 3A
YCR16w
delta
gly
tRNA
PGK1
136,155
Sau 3A
136
137
138
139
140
141
142
143
144
145
chromosome III position (kb)

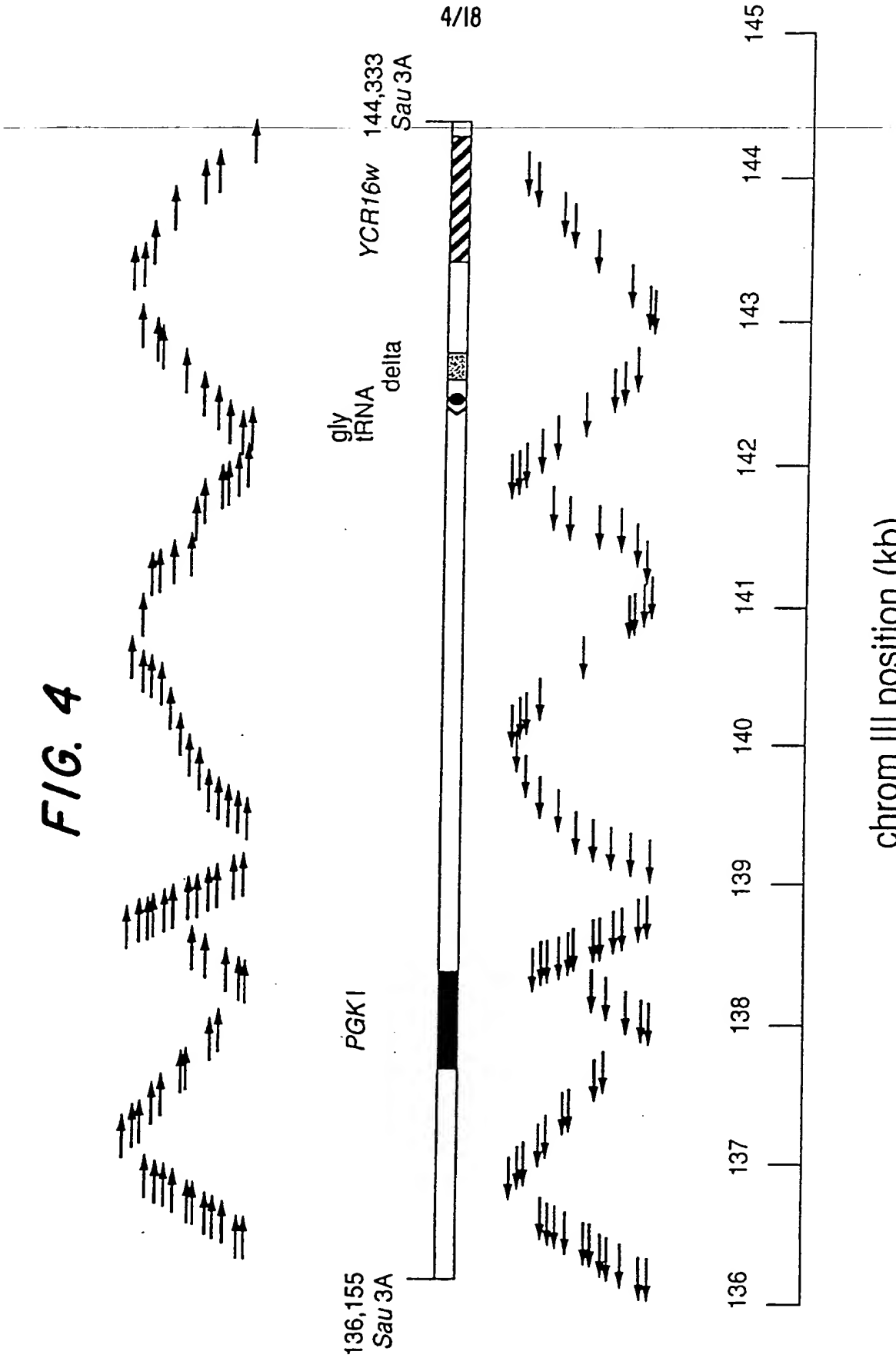

144,333
Sau 3A
YCR16w
gly
tRNA
delta
PGK1
136,155
Sau 3A
145
144
143
142
141
140
139
138
137
136
chrom III position (kb)

FIG. 4

FIG. 5

Classificiation of interval sizes for the 78 PART insertions into p76-2

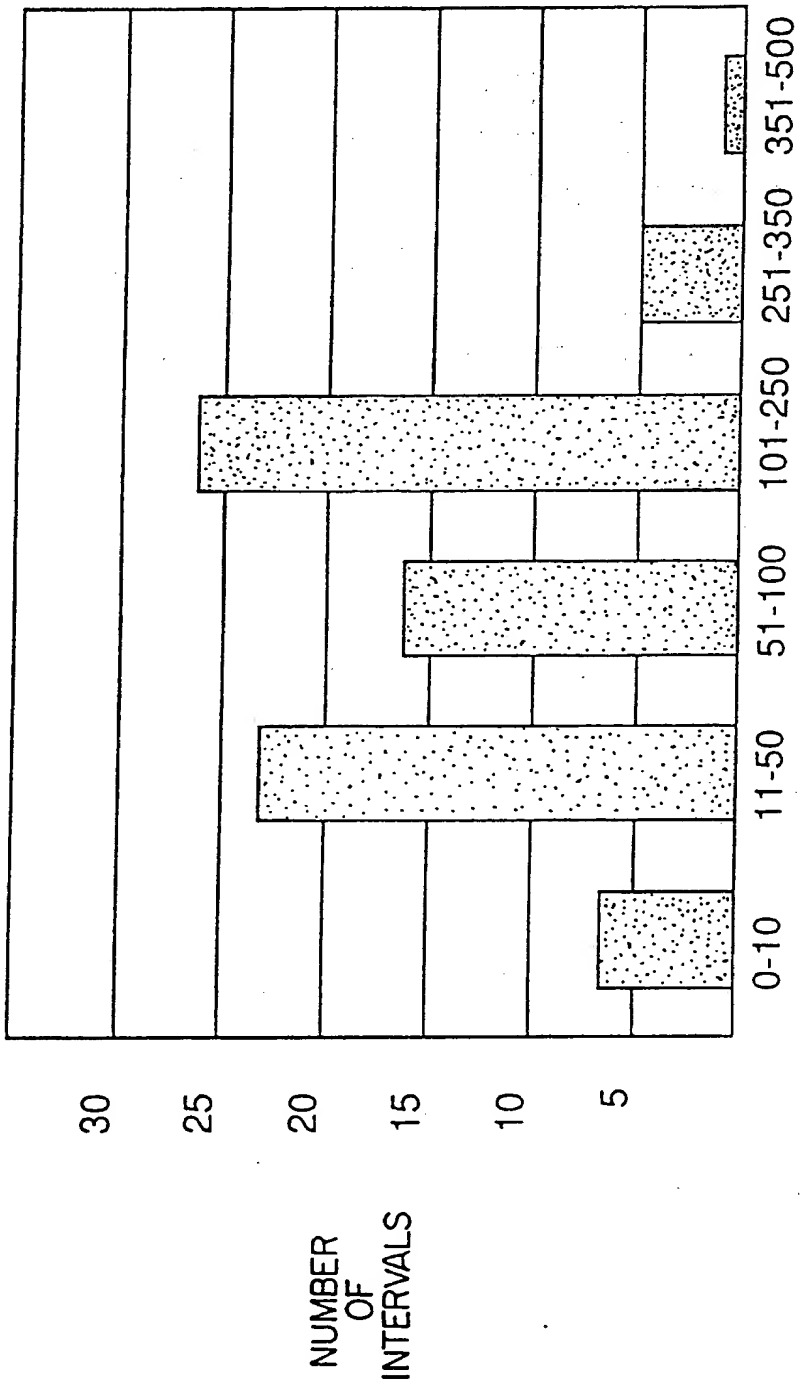
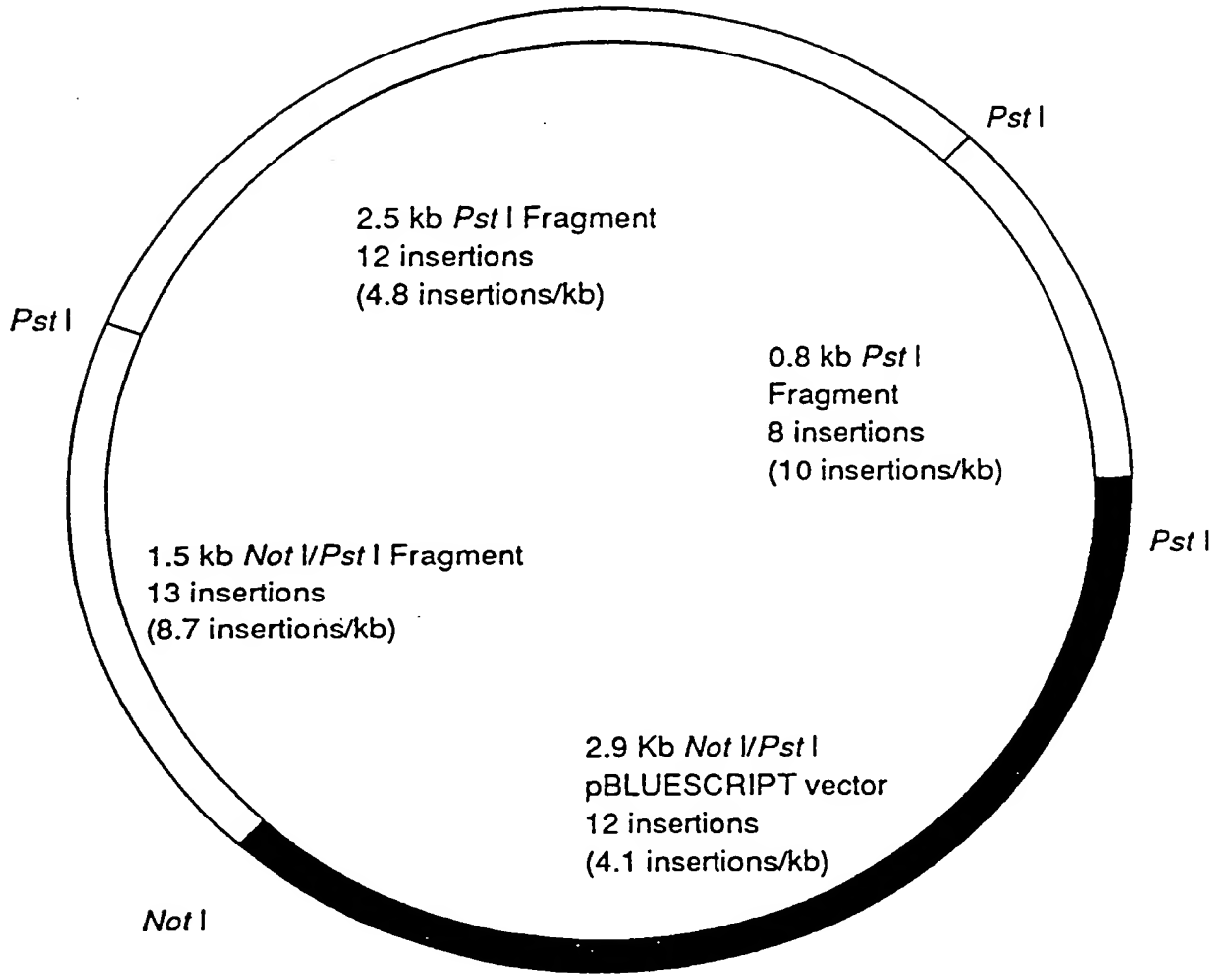


FIG. 6


Pst I
2.5 kb Pst I Fragment
12 insertions
(4.8 insertions/kb)
Pst I
0.8 kb Pst I
Fragment
8 insertions
(10 insertions/kb)
Pst I
1.5 kb Not I/Pst I Fragment
13 insertions
(8.7 insertions/kb)
2.9 Kb Not I/Pst I
pBLUESCRIPT vector
12 insertions
(4.1 insertions/kb)
Not I

pWAFp

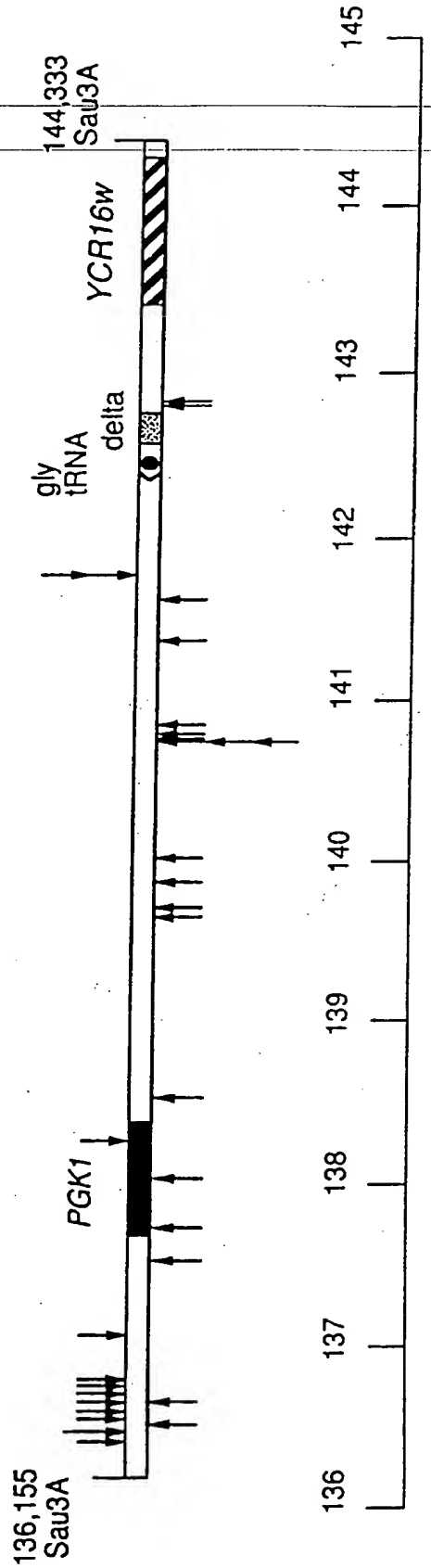

144,333
Sau3A
YCR16w
gly
tRNA
delta
PGK1
136,155
Sau3A
145
144
143
142
141
140
139
138
137
136
chrom III position (kb)

FIG. 7

FIG. 8A

```
                |  10        |  20        |  30        |  40        |  50        |  60
   1 TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG GAGACGGTCA  60
  61 CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG TCAGGGCGCG TCAGCGGGTG 120
 121 TTGGCGGGTG TCGGGGCTGG CTTAACTATG CGGCATCAGA GCAGATTGTA CTGAGAGTGC 180
 181 ACCATACCAC AGCTTTTCAA TTCAATTCAT CATTTTTTTT TTATTCTTTT TTTTGATTTC 240
 241 GGTTTCTTTG AAATTTTTTT GATTCGGTAA TCTCCGAACA GAAGGAAGAA CGAAGGAAGG 300
 301 AGCACAGACT TAGATTGGTA TATATACGCA TATGTAGTGT TGAAGAAACA TGAAATTGCC 360
 361 CAGTATTCTT AACCCAACTG CACAGAACAA AAACCTGCAG GAAACGAAGA TAAATCATGT 420
 421 CGAAAGCTAC ATATAAGGAA CGTGCTGCTA CTCATCCTAG TCCTGTTGCT GCCAAGCTAT 480
 481 TTAATATCAT GCACGAAAAG CAAACAAACT TGTGTGCTTC ATTGGATGTT CGTACCACCA 540
 541 AGGAATTACT GGAGTTAGTT GAAGCATTAG GTCCCAAAAT TTGTTTACTA AAAACACATG 600
 601 TGGATATCTT GACTGATTTT TCCATGGAGG GCACAGTTAA GCCGCTAAAG GCATTATCCG 660
 661 CCAAGTACAA TTTTTTACTC TTCGAAGACA GAAAATTTGC TGACATTGGT AATACAGTCA 720
 721 AATTGCAGTA CTCTGCGGGT GTATACAGAA TAGCAGAATG GGCAGACATT ACGAATGCAC 780
 781 ACGGTGTGGT GGGCCCAGGT ATTGTTAGCG GTTTGAAGCA GGCGGCAGAA GAAGTAACAA 840
 841 AGGAACCTAG AGGCCTTTTG ATGTTAGCAG AATTGTCATG CAAGGGCTCC CTATCTACTG 900
 901 GAGAATATAC TAAGGGTACT GTTGACATTG CGAAGAGCGA CAAAGATTTT GTTATCGGCT 960
 961 TTATTGCTCA AAGAGACATG GGTGGAAGAG ATGAAGGTTA CGATTGGTTG ATTATGACAC 1020
1021 CCGGTGTGGG TTTAGATGAC AAGGGAGACG CATTGGGTCA ACAGTATAGA ACCGTGGATG 1080
1081 ATGTGGTCTC TACAGGATCT GACATTATTA TTGTTGGAAG AGGACTATTT GCAAAGGGAA 1140
1141 GGGATGCTAA GGTAGAGGGT GAACGTTACA GAAAAGCAGG CTGGGAAGCA TATTTGAGAA 1200
1201 GATGCGGCCA GCAAAACTAA AAAACTGTAT TATAAGTAAA TGCATGTATA CTAAACTCAC 1260
1261 AAATTAGAGC TTCAATTTAA TTATATCAGT TATTACCCTA TGCGGTGTGA AATACCGCAC 1320
1321 AGATGCGTAA GGAGAAAATA CCGCATCAGG AAATTGTAAA CGTTAATATT TTGTTAAAAT 1380
1381 TCGCGTTAAA TTTTTGTTAA ATCAGCTCAT TTTTTAACCA ATAGGCCGAA ATCGGCAAAA 1440
1441 TCCCTTATAA ATCAAAAGAA TAGACCGAGA TAGGGTTGAG TGTTGTTCCA GTTTGGAACA 1500
1501 AGAGTCCACT ATTAAAGAAC GTGGACTCCA ACGTCAAAGG GCGAAAAACC GTCTATCAGG 1560
1561 GCGATGGCCC ACTACGTGAA CCATCACCCT AATCAAGTTT TTTGGGGTCG AGGTGCCGTA 1620
1621 AAGCACTAAA TCGGAACCCT AAAGGGAGCC CCCGATTTAG AGCTTGACGG GGAAAGCCGG 1680
1681 CGAACGTGGC GAGAAAGGAA GGGAAGAAAG CGAAAGGAGC GGGCGCTAGG GCGCTGGCAA 1740
1741 GTGTAGCGGT CACGCTGCGC GTAACCACCA CACCCGCCGC GCTTAATGCG CCGCTACAGG 1800
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FIG. 8B

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1801 GCGCGTCGCG CCATTCGCCA TTCAGGCTGC GCAACTGTTG GGAAGGGCGA TCGGTGCGGG 1860
1861 CCTCTTCGCT ATTACGCCAG CTGGCGAAAG GGGGATGTGC TGCAAGGCGA TTAAGTTGGG 1920
1921 TAACGCCAGG GTTTTCCCAG TCACGACGTT GTAAAACGAC GGCCAGTGAA TTGTAATACG 1980
1981 ACTCACTATA GGGCGAATTG GAGCTCGAAC ATGTTCACCG CGGTGGCGGC CGCTCTAGAA 2040
2041 CTAGTGGATC CCCCGGGCTG CAGGAATTCG ATATCAAGCT TATCGATACC GTCGACCTCG 2100
2101 AGAACATGTT CGGTACCAGC TTTTGTTCCC TTTAGTGAGG GTTAATTCCG AGCTTGGCGT 2160
2161 AATCATGGTC ATAGCTGTTT CCTGTGTGAA ATTGTTATCC GCTCACAATT CCACACAACA 2220
2221 TACGAGCCGG AAGCATAAAG TGTAAAGCCT GGGGTGCCTA ATGAGTGAGG TAACTCACAT 2280
2281 TAATTGCGTT GCGCTCACTG CCCGCTTTCC AGTCGGGAAA CCTGTCGTGC CAGCTGCATT 2340
2341 AATGAATCGG CCAACGCGCG GGGAGAGGCG GTTTGCGTAT TGGGCGCTCT TCCGCTTCCT 2400
2401 CGCTCACTGA CTCGCTGCGC TCGGTCGTTC GGCTGCGGCG AGCGGTATCA GCTCACTCAA 2460
2461 AGGCGGTAAT ACGGTTATCC ACAGAATCAG GGGATAACGC AGGAAAGAAC ATGTGAGCAA 2520
2521 AAGGCCAGCA AAAGGCCAGG AACCGTAAAA AGGCCGCGTT GCTGGCGTTT TTCCATAGGC 2580
2581 TCCGCCCCCC TGACGAGCAT CACAAAAATC GACGCTCAAG TCAGAGGTGG CGAAACCCGA 2640
2641 CAGGACTATA AAGATACCAG GCGTTTCCCC CTGGAAGCTC CCTCGTGCGC TCTCCTGTTC 2700
2701 CGACCCTGCC GCTTACCGGA TACCTGTCCG CCTTTCTCCC TTCGGGAAGC GTGGCGCTTT 2760
2761 CTCATAGCTC ACGCTGTAGG TATCTCAGTT CGGTGTAGGT CGTTCGCTCC AAGCTGGGCT 2820
2821 GTGTGCACGA ACCCCCCGTT CAGCCCGACC GCTGCGCCTT ATCCGGTAAC TATCGTCTTG 2880
2881 AGTCCAACCC GGTAAGACAC GACTTATCGC CACTGGCAGC AGCCACTGGT AACAGGATTA 2940
2941 GCAGAGCGAG GTATGTAGGC GGTGCTACAG AGTTCTTGAA GTGGTGGCCT AACTACGGCT 3000
3001 ACACTAGAAG GACAGTATTT GGTATCTGCG CTCTGCTGAA GCCAGTTACC TTCGGAAAAA 3060
3061 GAGTTGGTAG CTCTTGATCC GGCAAACAAA CCACCGCTGG TAGCGGTGGT TTTTTTGTTT 3120
3121 GCAAGCAGCA GATTACGCGC AGAAAAAAAG GATCTCAAGA AGATCCTTTG ATCTTTTCTA 3180
3181 CGGGGTCTGA CGCTCAGTGG AACGAAAACT CACGTTAAGG GATTTTGGTC ATGAGATTAT 3240
3241 CAAAAAGGAT CTTCACCTAG ATCCTTTTAA ATTAAAAATG AAGTTTTAAA TCAATCTAAA 3300
3301 GTATATATGA GTAAACTTGG TCTGACAGTT ACCAATGCTT AATCAGTGAG GCACCTATCT 3360
3361 CAGCGATCTG TCTATTTCGT TCATCCATAG TTGCCTGACT CCCCGTCGTG TAGATAACTA 3420
3421 CGATACGGGA GGGCTTACCA TCTGGCCCCA GTGCTGCAAT GATACCGATT ATTGAAGCAT 3480
3481 TTATCAGGGT TATTGTCTCA TGAGCGGATA CATATTTGAA TGTATTTAGA AAAATAAACA 3540
3541 AATAGGGGTT CCGCGCACAT TTCCCCGAAA AGTGCCACCT GGGTCCTTTT CATCACGTGC 3600
3601 TATAAAAATA ATTATAATTT AAATTTTTTA ATATAAATAT ATAAATTAAA AATAGAAAGT 3660
```

FIG. 8C

```
3661 AAAAAAAGAA ATTAAAGAAA AAATAGTTTT TGTTTTCCGA AGATGTAAAA GACTCTAGGG 3720
3721 GGATCGCCAA CAAATACTAC CTTTTATCTT GCTCTTCCTG CTCTCAGGTA TTAATGCCGA 3780
3781 ATTGTTTCAT CTTGTCTGTG TAGAAGACCA CACACGAAAA TCCTGTGATT TTACATTTTA 3840
3841 CTTATCGTTA ATCGAATGTA TATCTATTTA ATCTGCTTTT CTTGTCTAAT AAATATATAT 3900
3901 GTAAAGTACG CTTTTTGTTG AAATTTTTTA AACCTTTGTT TATTTTTTTT TCTTCATTCC 3960
3961 GTAACTCTTC TACCTTCTTT ATTTACTTTC TAAAATCCAA ATACAAAACA TAAAAATAAA 4020
4021 TAAACACAGA GTAAATTCCC AAATTATTCC ATCATTAAAA GATACGAGGC GCGTGTAAGT 4080
4081 TACAGGCAAG CGATCCGTCC TAAGAAACCA TTATTATCAT GACATTAACC TATAAAAATA 4140
4141 GGCGTATCAC GAGGCCCTTT CGTC                                      4164

          |  10        |  20        |  30        |  40        |  50        |  60
```

DNA Strider™ 1.2 ### Tuesday, March 1, 1994 1:25:54 PM

pAT-2 -> List

DNA sequence 4933 b.p. TCGCGCGTTTCG ... GGCCCTTTCGTC circular

Friday, February 11, 1994 derivative of pSD528, Tyi mcs with dhfr in BamHI, EcoRI PART insert liberated with XmnI

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  61 CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG TCAGGGCGCG TCAGCGGGTG 120
 121 TTGGCGGGTG TCGGGGCTGG CTTAACTATG CGGCATCAGA GCAGATTGTA CTGAGAGTGC 180
 181 ACCATACCAC AGCTTTTCAA TTCAATTCAT CATTTTTTTT TTATTCTTTT TTTTGATTTC 240
 241 GGTTTCTTTG AAATTTTTTT GATTCGGTAA TCTCCGAACA GAAGGAAGAA CGAAGGAAGG 300
 301 AGCACAGACT TAGATTGGTA TATATACGCA TATGTAGTGT TGAAGAAACA TGAAATTGCC 360
 361 CAGTATTCTT AACCCAACTG CACAGAACAA AAACCTGCAG GAAACGAAGA TAAATCATGT 420
 421 CGAAAGCTAC ATATAAGGAA CGTGCTGCTA CTCATCCTAG TCCTGTTGCT GCCAAGCTAT 480
 481 TTAATATCAT GCACGAAAAG CAAACAAACT TGTGTGCTTC ATTGGATGTT CGTACCACCA 540
 541 AGGAATTACT GGAGTTAGTT GAAGCATTAG GTCCCAAAAT TTGTTTACTA AAAACACATG 600
 601 TGGATATCTT GACTGATTTT TCCATGGAGG GCACAGTTAA GCCGCTAAAG GCATTATCCG 660
 661 CCAAGTACAA TTTTTTACTC TTCGAAGACA GAAAATTTGC TGACATTGGT AATACAGTCA 720
 721 AATTGCAGTA CTCTGCGGGT GTATACAGAA TAGCAGAATG GGCAGACATT ACGAATGCAC 780
 781 ACGGTGTGGT GGGCCCAGGT ATTGTTAGCG GTTTGAAGCA GGCGGCAGAA GAAGTAACAA 840
 841 AGGAACCTAG AGGCCTTTTG ATGTTAGCAG AATTGTCATG CAAGGGCTCC CTATCTACTG 900
 901 GAGAATATAC TAAGGGTACT GTTGACATTG CGAAGAGCGA CAAAGATTTT GTTATCGGCT 960
 961 TTATTGCTCA AAGAGACATG GGTGGAAGAG ATGAAGGTTA CGATTGGTTG ATTATGACAC 1020
1021 CCGGTGTGGG TTTAGATGAC AAGGGAGACG CATTGGGTCA ACAGTATAGA ACCGTGGATG 1080
1081 ATGTGGTCTC TACAGGATCT GACATTATTA TTGTTGGAAG AGGACTATTT GCAAAGGGAA 1140
1141 GGGATGCTAA GGTAGAGGGT GAACGTTACA GAAAAGCAGG CTGGGAAGCA TATTTGAGAA 1200
1201 GATGCGGCCA GCAAAACTAA AAAACTGTAT TATAAGTAAA TGCATGTATA CTAAACTCAC 1260
1261 AAATTAGAGC TTCAATTTAA TTATATCAGT TATTACCCTA TGCGGTGTGA AATACCGCAC 1320
1321 AGATGCGTAA GGAGAAAATA CCGCATCAGG AAATTGTAAA CGTTAATATT TTGTTAAAAT 1380
1381 TCGCGTTAAA TTTTTGTTAA ATCAGCTCAT TTTTTAACCA ATAGGCCGAA ATCGGCAAAA 1440
1441 TCCCTTATAA ATCAAAAGAA TAGACCGAGA TAGGGTTGAG TGTTGTTCCA GTTTGGAACA 1500
1501 AGAGTCCACT ATTAAAGAAC GTGGACTCCA ACGTCAAAGG GCGAAAAACC GTCTATCAGG 1560
1561 GCGATGGCCC ACTACGTGAA CCATCACCCT AATCAAGTTT TTTGGGGTCG AGGTGCCGTA 1620
1621 AAGCACTAAA TCGGAACCCT AAAGGGAGCC CCCGATTTAG AGCTTGACGG GGAAAGCCGG 1680
1681 CGAACGTGGC GAGAAAGGAA GGGAAGAAAG CGAAAGGAGC GGGCGCTAGG GCGCTGGCAA 1740
1741 GTGTAGCGGT CACGCTGCGC GTAACCACCA CACCCGCCGC GCTTAATGCG CCGCTACAGG 1800
1801 GCGCGTCGCG CCATTCGCCA TTCAGGCTGC GCAACTGTTG GGAAGGGCGA TCGGTGCGGG 1860
1861 CCTCTTCGCT ATTACGCCAG CTGGCGAAAG GGGGATGTGC TGCAAGGCGA TTAAGTTGGG 1920
1921 TAACGCCAGG GTTTTCCCAG TCACGACGTT GTAAAACGAC GGCCAGTGAA TTGTAATACG 1980
1981 ACTCACTATA GGGCGAATTG GAGCTCGAAC ATGTTCACCG CGGTGGCGGC CGCTCTAGAA 2040
2041 CTAGTGGATC CTGCAAGCAG GATAGACGGC ATGCACGATT TGTAATAACA GAGTGTCTTG 2100
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2161 GGGTGACAAA ACGAGCATGC TTACTAATAA AATGTTAACC TCTGAGGAAG AATTGTGAAA 2220
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2281 TGGAGTGCCA AAGGTGAACA GCTCCTGTTT AAAGCTATTA CCTATAACCA ATGGCTGTTG 2340
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2401 ACACGTTCAA GTTTTACATC TGACAATGAG AACGTATTGA TCTTTCCATC AATTAAAGAT 2460
2461 GCTTTAACCA ACCTAAAGAA AATAACGGAT CATGTCATTG TTTCAGGTGG TGGGGAGATA 2520
2521 TACAAAAGCC TGATCGATCA AGTAGATACA CTACATATAT CTACAATAGA CATCGAGCCG 2580
2581 GAAGGTGATG TTTACTTTCC TGAAATCCCC AGCAATTTTA GGCCAGTTTT TACCCAAGAC 2640
2641 TTCGCCTCTA ACATAAATTA TAGTTACCAA ATCTGGCAAA AGGGTTAACA AGTGGCAGCA 2700
2701 ACGGATTCGC AAACCTGTCA CGCCTTTTGT GCCAAAAGCC GCGCCAGGTT TGCGATCCGC 2760
2761 TGTGCCAGGC GTTAGGCGTC ATATGAAGAT TTCGGTGATC CCTGAGCAGG TGGCGGAAAC 2820
2821 ATTGGATGCT GAGAATTCGA TATCAAGCTT ATCGATACCG TCGACCTCGA GAACATGTTC 2880
2881 GGTACCAGCT TTTGTTCCCT TTAGTGAGGG TTAATTCCGA GCTTGGCGTA ATCATGGTCA 2940
2941 TAGCTGTTTC CTGTGTGAAA TTGTTATCCG CTCACAATTC CACACAACAT ACGAGCCGGA 3000
3001 AGCATAAAGT GTAAAGCCTG GGGTGCCTAA TGAGTGAGGT AACTCACATT AATTGCGTTG 3060
3061 CGCTCACTGC CCGCTTTCCA GTCGGGAAAC CTGTCGTGCC AGCTGCATTA ATGAATCGGC 3120
3121 CAACGCGCGG GGAGAGGCGG TTTGCGTATT GGGCGCTCTT CCGCTTCCTC GCTCACTGAC 3180
3181 TCGCTGCGCT CGGTCGTTCG GCTGCGGCGA GCGGTATCAG CTCACTCAAA GGCGGTAATA 3240
3241 CGGTTATCCA CAGAATCAGG GGATAACGCA GGAAAGAACA TGTGAGCAAA AGGCCAGCAA 3300
3301 AAGGCCAGGA ACCGTAAAAA GGCCGCGTTG CTGGCGTTTT TCCATAGGCT CCGCCCCCCT 3360
3361 GACGAGCATC ACAAAAATCG ACGCTCAAGT CAGAGGTGGC GAAACCCGAC AGGACTATAA 3420
3421 AGATACCAGG CGTTTCCCCC TGGAAGCTCC CTCGTGCGCT CTCCTGTTCC GACCCTGCCG 3480
3481 CTTACCGGAT ACCTGTCCGC CTTTCTCCCT TCGGGAAGCG TGGCGCTTTC TCATAGCTCA 3540
3541 CGCTGTAGGT ATCTCAGTTC GGTGTAGGTC GTTCGCTCCA AGCTGGGCTG TGTGCACGAA 3600
```

FIGURE 9A

FIG. 9B

```
1801 GCGCGTCGCG CCATTCGCCA TTCAGGCTGC GCAACTGTTG GGAAGGGCGA TCGGTGCGGG 1860
1861 CCTCTTCGCT ATTACGCCAG CTGGCGAAAG GGGGATGTGC TGCAAGGCGA TTAAGTTGGG 1920
1921 TAACGCCAGG GTTTTCCCAG TCACGACGTT GTAAAACGAC GGCCAGTGAA TTGTAATACG 1980
1981 ACTCACTATA GGGCGAATTG GAGCTCGAAC ATGTTCACCG CGGTGGCGGC CGCTCTAGAA 2040
2041 CTAGTGGATC CTGCAAGCAG GATAGACGGC ATGCACGATT TGTAATAACA GAGTGTCTTG 2100
2101 TATTTTTAAA GAAAGTCTAT TTAATACAAG TGATTATATT AATTAACGGT AAGCATCAGC 2160
2161 GGGTGACAAA ACGAGCATGC TTACTAATAA AATGTTAACC TCTGAGGAAG AATTGTGAAA 2220
2221 CTATCACTAA TGGTAGCTAT ATCGAAGAAT GGAGTTATCG GGAATGGCCC TGATATTCCA 2280
2281 TGGAGTGCCA AAGGTGAACA GCTCCTGTTT AAAGCTATTA CCTATAACCA ATGGCTGTTG 2340
2341 GTTGGACGCA AGACTTTTGA ATCAATGGGA GCATTACCCA ACCGAAAGTA TGCGGTCGTA 2400
2401 ACACGTTCAA GTTTTACATC TGACAATGAG AACGTATTGA TCTTTCCATC AATTAAGAT 2460
2461 GCTTTAACCA ACCTAAAGAA AATAACGGAT CATGTCATTG TTTCAGGTGG TGGGGAGATA 2520
2521 TACAAAAGCC TGATCGATCA AGTAGATACA CTACATATAT CTACAATAGA CATCGAGCCG 2580
2581 GAAGGTGATG TTTACTTTCC TGAAATCCCC AGCAATTTTA GGCCAGTTTT TACCCAAGAC 2640
2641 TTCGCCTCTA ACATAAATTA TAGTTACCAA ATCTGGCAAA AGGGTTAACA AGTGGCAGCA 2700
2701 ACGGATTCGC AAACCTGTCA CGCCTTTTGT GCCAAAAGCC GCGCCAGGTT TGCGATCCGC 2760
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3181 TCGCTGCGCT CGGTCGTTCG GCTGCGGCGA GCGGTATCAG CTCACTCAAA GGCGGTAATA 3240
3241 CGGTTATCCA CAGAATCAGG GGATAACGCA GGAAAGAACA TGTGAGCAAA AGGCCAGCAA 3300
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3421 AGATACCAGG CGTTTCCCCC TGGAAGCTCC CTCGTGCGCT CTCCTGTTCC GACCCTGCCG 3480
3481 CTTACCGGAT ACCTGTCCGC CTTTCTCCCT TCGGGAAGCG TGGCGCTTTC TCATAGCTCA 3540
3541 CGCTGTAGGT ATCTCAGTTC GGTGTAGGTC GTTCGCTCCA AGCTGGGCTG TGTGCACGAA 3600
```

FIG. 9C

```
3601 CCCCCCGTTC AGCCCGACCG CTGCGCCTTA TCCGGTAACT ATCGTCTTGA GTCCAACCCG 3660
3661 GTAAGACACG ACTTATCGCC ACTGGCAGCA GCCACTGGTA ACAGGATTAG CAGAGCGAGG 3720
3721 TATGTAGGCG GTGCTACAGA GTTCTTGAAG TGGTGGCCTA ACTACGGCTA CACTAGAAGG 3780
3781 ACAGTATTTG GTATCTGCGC TCTGCTGAAG CCAGTTACCT TCGGAAAAAG AGTTGGTAGC 3840
3841 TCTTGATCCG GCAACAAAC CACCGCTGGT AGCGGTGGTT TTTTTGTTTG CAAGCAGCAG 3900
3901 ATTACGCGCA GAAAAAAGG ATCTCAAGAA GATCCTTTGA TCTTTTCTAC GGGTCTGACC 3960
3961 GCTCAGTGGA ACGAAAACTC ACGTTAAGGG ATTTTGGTCA TGAGATTATC AAAAAGGATC 4020
4021 TTCACCTAGA TCCTTTTAAA TTAAAAATGA AGTTTTAAAT CAATCTAAAG TATATATGAG 4080
4081 TAAACTTGGT CTGACAGTTA CCAATGCTTA ATCAGTGAGG CACCTATCTC AGCGATCTGT 4140
4141 CTATTTCGTT CATCCATAGT TGCCTGACTC CCCGTCGTGT AGATAACTAC GATACGGGAG 4200
4201 GGCTTACCAT CTGGCCCCAG TGCTGCAATG ATACCGATTA TTGAAGCATT TATCAGGGTT 4260
4261 ATTGTCTCAT GAGCGGATAC ATATTTGAAT GTATTTAGAA AAATAAACAA ATAGGGGTTC 4320
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4381 TTATAATTTA AATTTTTTAA TATAAATATA TAAATTAAAA ATAGAAAGTA AAAAAGAAA 4440
4441 TTAAAGAAAA AATAGTTTTT GTTTTCCGAA GATGTAAAAG ACTCTAGGGG GATCGCCAAC 4500
4501 AAATACTACC TTTTATCTTG CTCTTCCTGC TCTCAGGTAT TAATGCCGAA TTGTTTCATC 4560
4561 TTGTCTGTGT AGAAGACCAC ACACGAAAAT CCTGTGATTT TACATTTTAC TTATCGTTAA 4620
4621 TCGAATGTAT ATCTATTTAA TCTGCTTTTC TTGTCTAATA AATATATATG TAAAGTACGC 4680
4681 TTTTTGTTGA AATTTTTTAA ACCTTTGTTT ATTTTTTTTT CTTCATTCCG TAACTCTTCT 4740
4741 ACCTTCTTTA TTTACTTTCT AAAATCCAAA TACAAAACAT AAAAATAAAT AAACACAGAG 4800
4801 TAAATTCCCA AATTATTCCA TCATTAAAAG ATACGAGGCG CGTGTAAGTT ACAGGCAAGC 4860
4861 GATCCGTCCT AAGAAACCAT TATTATCATG ACATTAACCT ATAAAAATAG GCGTATCACG 4920
4921 AGGCCCTTTC GTC                                                    4933
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```

DNA Strider™ 1.2 ### Tuesday, March 1, 1994 1:17:57 PM

PART from pAT-2 -> List

DNA sequence 664 b.p. TGTTCACCGCGG ... ACCTCGAGAACA linear

Complete PART sequence cleaved from pAT-2 upon digestion with Xmn I

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 61 TGCACGATTT GTAATAACAG AGTGTCTTGT ATTTTTAAAG AAAGTCTATT TAATACAAGT 120
121 GATTATATTA ATTAACGGTA AGCATCAGCG GGTGACAAAA CGAGCATGCT TACTAATAAA 180
181 ATGTTAACCT CTGAGGAAGA ATTGTGAAAC TATCACTAAT GGTAGCTATA TCGAAGAATG 240
241 GAGTTATCGG GAATGGCCCT GATATTCCAT GGAGTGCCAA AGGTGAACAG CTCCTGTTTA 300
301 AAGCTATTAC CTATAACCAA TGGCTGTTGG TTGGACGCAA GACTTTTGAA TCAATGGGAG 360
361 CATTACCCAA CCGAAAGTAT GCGGTCGTAA CACGTTCAAG TTTTACATCT GACAATGAGA 420
421 ACGTATTGAT CTTTCCATCA ATTAAAGATG CTTTAACCAA CCTAAAGAAA ATAACGGATC 480
481 ATGTCATTGT TTCAGGTGGT GGGGAGATAT ACAAAAGCCT GATCGATCAA GTAGATACAC 540
541 TACATATATC TACAATAGAC ATCGAGCCGG AAGGTGATGT TTACTTTCCT GAAATCCCCA 600
601 GCAATTTTAG GCCAGTTTTT ACCCAAGACT TCGCCTCTAA CATAAATTAT AGTTACCAAA 660
661 TCTGGCAAAA GGGTTAACAA GTGGCAGCAA CGGATTCGCA AACCTGTCAC GCCTTTTGTG 720
721 CCAAAAGCCG CGCCAGGTTT GCGATCCGCT GTGCCAGGCG TTAGGCGTCA TATGAAGATT 780
781 TCGGTGATCC CTGAGCAGGT GGCGGAAACA TTGGATGCTG AGAATTCGAT ATCAAGCTTA 840
841 TCGATACCGT CGACCTCGAG AACA                                        664
        |  10     |  20     |  30     |  40     |  50     |  60
```
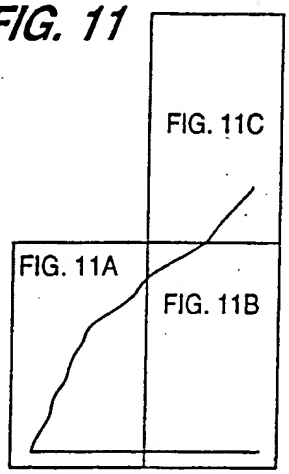

FIGURE 10

FIG. 11A

FIG. 11



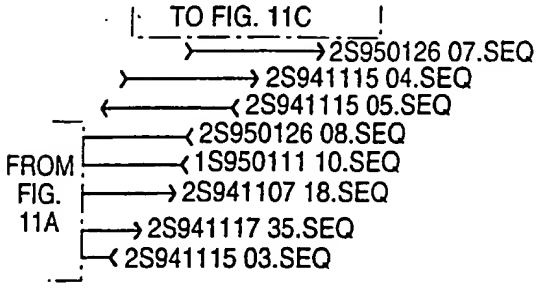
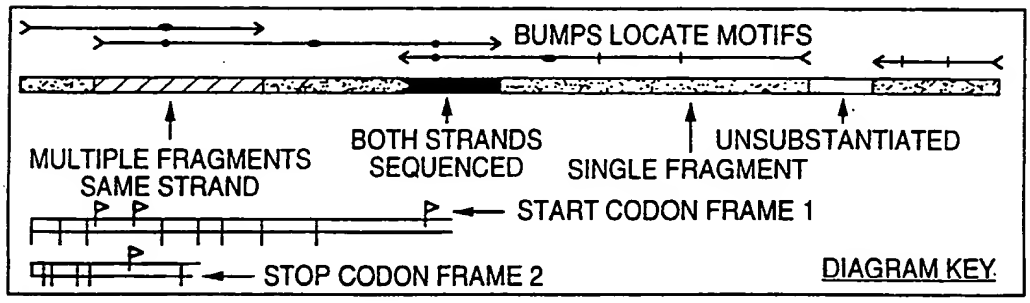

TO FIG. 11C
2S950126 07.SEQ
2S941115 04.SEQ
2S941115 05.SEQ
2S950126 08.SEQ
FROM FIG. 11A
1S950111 10.SEQ
2S941107 18.SEQ
2S941117 35.SEQ
2S941115 03.SEQ

FIG. 11B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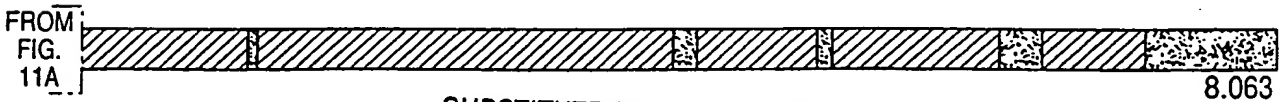
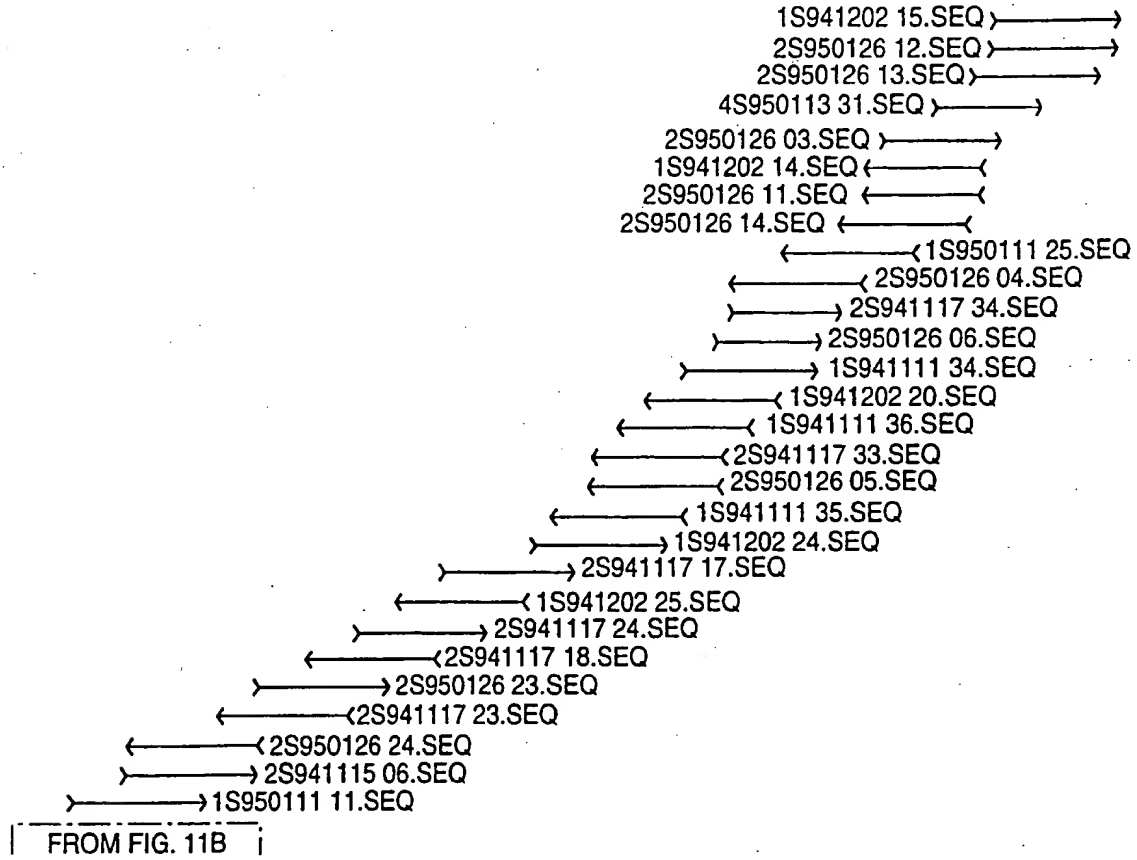
MULTIPLE FRAGMENTS SAME STRAND
BOTH STRANDS SEQUENCED
SINGLE FRAGMENT
UNSUBSTANTIATED
START CODON FRAME 1
STOP CODON FRAME 2
DIAGRAM KEY


FROM FIG. 11A

8.063

FIG. 11C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2S950126 13.SEQ
4S950113 31.SEQ
2S950126 03.SEQ
1S941202 14.SEQ
2S950126 11.SEQ
2S950126 14.SEQ
1S950111 25.SEQ
2S950126 04.SEQ
2S941117 34.SEQ
2S950126 06.SEQ
1S941111 34.SEQ
1S941202 20.SEQ
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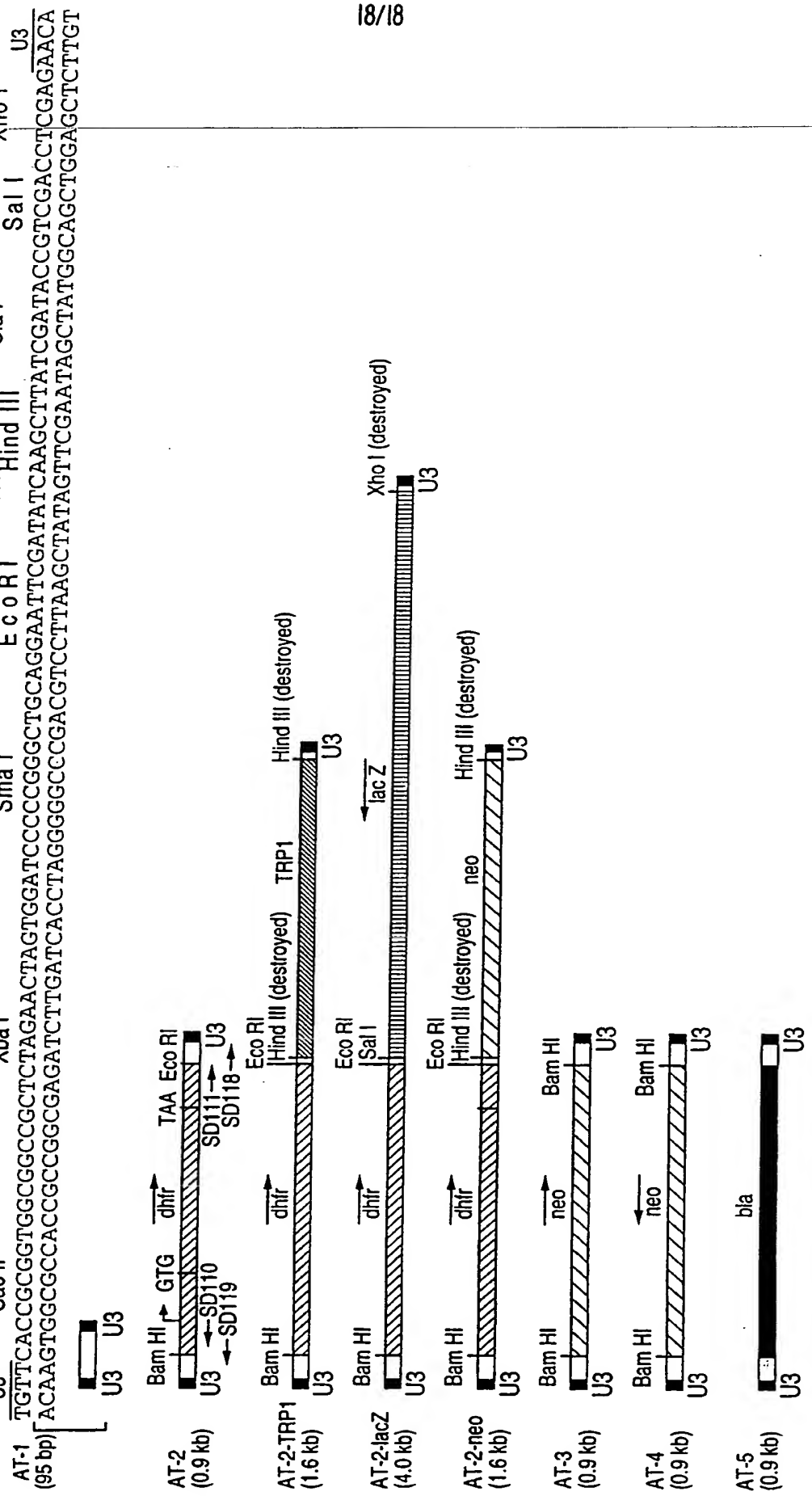
2S941117 33.SEQ
2S950126 05.SEQ
1S941111 35.SEQ
1S941202 24.SEQ
2S941117 17.SEQ
1S941202 25.SEQ
2S941117 24.SEQ
2S941117 18.SEQ
2S950126 23.SEQ
2S941117 23.SEQ
2S950126 24.SEQ
2S941115 06.SEQ
1S950111 11.SEQ
FROM FIG. 11B

FIG. 12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ACAAGTGGCGCCACCGCCGGCGAGATCTTGATCACCTAGGGGGCCCGACGTCCTTAAGCTATAGTTCGAATAGCTATGGCAGCTGGAGCTCTTGT
Not I
Sac II
Xba I
Bam HI
Sma I
Pst I
E c o R I
Eco RV
Hind III
Cla I
Sal I
Xho I
U3
AT-1
(95 bp)
U3
U3
AT-2
(0.9 kb)
Bam HI
GTG
dhfr
TAA
Eco RI
U3
SD110
SD119
SD111
SD118
U3
AT-2-TRP1
(1.6 kb)
Bam HI
dhfr
Eco RI
Hind III (destroyed)
TRP1
Hind III (destroyed)
U3
AT-2-lacZ
(4.0 kb)
Bam HI
dhfr
Eco RI
Sal I
lac Z
Xho I (destroyed)
U3
AT-2-neo
(1.6 kb)
Bam HI
dhfr
Eco RI
Hind III (destroyed)
neo
Hind III (destroyed)
U3
AT-3
(0.9 kb)
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neo
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U3
AT-4
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Bam HI
neo
Bam HI
U3
AT-5
(0.9 kb)
bla
U3

INTERNATIONAL SEARCH REPORT

Internat' Application No
PCT/US 95/02520

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C12N15/90

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US,A,5 212 080 (NAG) 18 May 1993 see the whole document ---	1,12,23, 31,36, 44,45
A	US,A,5 137 829 (NAG) 11 August 1992 see the whole document ---	1,12,23, 31,36, 44,45
A	US,A,5 227 288 (BLETTNER) 13 July 1993 see the whole document ---	1,12,23, 31
A	WO,A,90 00621 (RIJKSUNIVERSITEIT TE LEIDEN) 25 January 1990 see the whole document --- -/--	1,12,23, 31

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 June 1995	27. 06. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Osborne, H

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,92 03578 (UNITED STATES OF AMERICA) 5 March 1992 see the whole document ---	1,12,23, 31,36, 44,45
A	GENES AND DEVELOPMENT, vol. 4, March 1990 pages 324-330, EICHINGER, D ET AL 'A specific terminal structure is required for Ty1 transposition' see the whole document ---	36-45
A	CELL, vol. 49, May 1987 pages 347-356, BROWN, F. ET AL 'correct integration of retroviral DNA invitro' see the whole document -----	36-45

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internal Application No
PCT/US 95/02520

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-5212080	18-05-93	US-A- 5137829	11-08-92
		US-A- 5316946	31-05-94
US-A-5137829	11-08-92	US-A- 5316946	31-05-94
		US-A- 5212080	18-05-93
US-A-5227288	13-07-93	NONE	
WO-A-9000621	25-01-90	NL-A- 8801805	01-02-90
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